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14. ABSTRACT The original purpose of this research, as proposed in the statement of work, was to determine the mechanism by which prostate cancer (PCa) cells become resistant to the anti-tumor activity of vitamin D. The proposal focused on a PCa-specific deficiency in a key vitamin D metabolizing enzyme, 1α-hydroxylase (1αOH). During the first year, we encountered unforeseen difficulties with one of the key techniques in the original proposal. Therefore we decided to focus on vitamin D target genes, whose expression would be effected downstream of 1αOH bioactivation of vitamin D. Using normal human prostatic epithelial cells and prostate cancer cell lines, we examined the role of map kinase phosphatase 5 (MKP5), a recently discovered target gene of vitamin D, in mediating anti-tumor activities. MKP5 dephosphorylates/inactivates the stress activated protein kinase p38. Interestingly, in the prostate cancer cell lines LNCaP, PC-3 and DU 145, 1,25D did not up-regulate MKP5 or inactivate p38. 1,25D inhibited both UV and inflammation-induced p38 phosphorylation and downstream IL-6 production in a MKP5-dependendt manner. As inflammation emerges as a risk factor for prostate cancer, there is potential for chemoprevention by anti-inflammatory agents. We proceeded to expand upon these results and focused on MKP5 as a mediator of potentially chemopreventive anti-inflammatory activities of other phytochemicals in the prostate. Curcumin, the phytochemical found in turmeric, up-regulated MKP5, subsequently decreasing cytokine-induced p38-dependent pro-inflammatory changes in normal prostatic epithelial cells. Resveratrol and [6]-gingerol, phytochemicals present in red wine and ginger, respectively, also up-regulated MKP5 in normal prostate epithelial cells. Moreover, we found that prostate cancer cell lines DU 145, PC-3, LNCaP and LAPC-4 retained the ability to up-regulate MKP5 following curcumin, resveratrol and [6]-gingerol exposure, suggesting utility of these phytochemicals in prostate cancer treatment. In summary, our findings show direct anti-inflammatory activity of MKP5 in prostate cells and suggest that up-regulation of MKP5 by phytochemicals may contribute to their chemopreventive actions by decreasing prostatic inflammation.

15. SUBJECT TERMS
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Introduction

The original purpose of this research, as proposed in the statement of work, was to determine the mechanism by which prostate cancer (PCa) cells become resistant to the anti-tumor activity of vitamin D. The experiments in the proposal were focused upon one particular aspect of PCa resistance to vitamin D, decreased enzymatic activity of vitamin D 1α-25-hydroxylase (1αOH). 1αOH is the enzyme that converts vitamin D to its active form, 1,25-dihydroxyvitamin D (1,25D). Since the ability of 1αOH to synthesize 1,25D has been shown to be impaired in PCa compared to normal prostate (Hsu et al. 2001), we hypothesized that this is a key mechanism by which PCa escapes growth control by 1,25D. The key assay necessary for us to investigate our hypothesis was the 1αOH enzymatic activity assay. Despite multiple attempts to troubleshoot the assay, I was unable to consistently measure 1αOH enzymatic activity. Since I was not able to measure 1αOH enzymatic activity itself, we decided to focus on expression changes in vitamin D target genes that would be effected downstream of 1αOH bioactivation of vitamin D. We investigated a newly discovered molecular target of vitamin D, MAP kinase phosphatase 5 (MKP5), whose expression is dose-dependently up-regulated by vitamin D. Furthermore, MKP5 expression appears to be diminished or absent in PCa cells. Although the experiments performed during this past 2 years differ slightly from the proposed experiments, they address the original purpose of the work, which was to elucidate the mechanisms by which PCa cells become resistant to the anti-tumor activity of vitamin D. This shift in the focus proved to be advantageous and led to two first author manuscripts in which we report the significance of our findings (appendix A and B).

Body/Results

Using cDNA microarrays, we had recently identified a new vitamin D-responsive gene, MAP kinase phosphatase 5 (MKP5) (Peehl et al. 2004). The mRNA expression of MKP5, also known as dual specificity phosphatase 10 (DUSP10), was consistently increased by 1,25D treatment of primary cultures of prostatic epithelial cells (Peehl et al. 2004). As a member of the dual specificity MKP (DS-MKP) family of proteins that dephosphorylate mitogen activated protein kinases (MAPKs), MKP5 dephosphorylates p38 and JNK, but not ERK (Tanoue et al. 1999; Theodosiou et al. 1999). The potential ability of vitamin D to inhibit p38 through MKP5 is of interest to PCa prevention since p38 is activated by oxidative stress, hypoxia and inflammation, all of which contribute to PCa development (Dennis et al. 2002; Hochachka et al. 2002; Palapattu et al. 2004; Roux and Blenis 2004). In particular, inflammation plays a causal role in the progression of many cancers including liver, bladder and gastric cancers (Coussens and Werb 2002) and a similar role for inflammation in the development of PCa is now emerging (De Marzo et al. 2004; Nelson et al. 2004; Palapattu et al. 2004). The strongest evidence linking inflammation to PCa is from the recent findings that show regular administration of non-steroidal anti-inflammatory drugs (NSAIDS) significantly decreases PCa risk in older men by 60-80% (Palapattu et al. 2004; Platz et al. 2005). Also, men with chronic and/or acute inflammation of the prostate, either in the form of prostatitis or a sexually transmitted disease, have an increased risk of developing PCa (Dennis et al. 2002; Fernandez et al. 2005).

Inhibitors of p38 are classically anti-inflammatory, suggesting that some of the activities attributed to vitamin D, including prostate cancer prevention, may be a result of p38 inhibition and decreased inflammation. One of the downstream consequences of p38 protein kinase pathway activation is an increase in pro-inflammatory cytokine production in order to amplify the inflammatory response (Brinkman et al. 1999; Park et al. 2003). Interleukin-6 (IL-6) is a p38-regulated pleiotropic cytokine that has been historically associated with PCa (Giri et al. 2001;

Corcoran and Costello 2003). Elevated levels of IL-6 are found in the serum of PCa patients and primary PCa tumors over-express IL-6 (Giri et al. 2001; Michalaki et al. 2004). IL-6 is also involved in the progression of PCa to androgen-independent PCa because it can facilitate androgen receptor signaling in the absence of androgens (Corcoran and Costello 2003; Culig 2004; Culig et al. 2005).

Since there is increasing evidence linking inflammation to PCa development, our studies focused on characterizing regulation of IL-6 production via p38 inhibition by 1,25D as a potentially significant cancer prevention activity. Using primary epithelial cell cultures (E-PZ) derived from the normal peripheral zone [the major site of origin of prostatic adenocarcinomas(McNeal et al. 1988)] as our model system, we characterized MKP5 induction by 1,25D and revealed MKP5 as the mediator of p38 kinase inhibition and decreased IL-6 production by 1,25D in normal prostatic epithelial cells. We also compared the ability of 1,25D to regulate MKP5 in prostate cancer-derived cells to the normal prostatic cells.

The materials, methods, results and figures are detailed in our submitted manuscript "Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D" (Appendix A.)

We next proceeded to expanded upon these results and focused on MKP5 as a mediator of potentially chemopreventive anti-inflammatory activities in the prostate. To evaluate the role of MKP5 in the inflammatory response of normal prostatic epithelial cells derived from the peripheral zone (E-PZ), it was necessary to identify a pro-inflammatory stimulus that signaled through p38. Co-treatment of cells with TNF*α* and IL-1*β* stimulated a robust increase in cytokine mRNA, COX-2 mRNA and protein, and NFκB activity in E-PZ cells, all of which could be blocked by SB202190 and hence were p38-dependent. Co-treatment with TNF*α* and IL-1*β* (T+I) was used as the inflammatory stimulus in all subsequent experiments.

Our next goal was to assess whether MKP5, as a p38 MAP kinase phosphatase, could inhibit or decrease the powerful pro-inflammatory molecular changes induced by TNF*α* and IL-1*β* stimulation in E-PZ cells. Transfection of E-PZ cells with pcDNA3.1-MKP5 increased MKP5 mRNA levels 10-50 fold at 24 hours and was able to block T+I –stimulated p38 phosphorylation, COX-2 and cytokine expression and NFκB-luc activity in a dose dependent manner. These results reveal that MKP5 has a significant role in reducing T+I –stimulated inflammatory signaling by inhibiting p38-mediated pro-inflammatory processes in normal prostatic E-PZ cells.

Curcumin is the polyphenolic compound present in the spice turmeric. Curcumin has been shown to have significant anti-inflammatory effects in a variety of cell types (Cho et al. 2005) and up-regulated MKP5 mRNA in a squamous cell carcinoma microarray study (Yan et al. 2005). We speculated that curcumin would have similar anti-inflammatory effects in the normal prostatic E-PZ cells and that these effects would be mediated by MKP5. In agreement with our hypothesis, curcumin dose-dependently up-regulated MKP5 mRNA and inhibited T+I-stimulated p38 phosphorylation, COX-2 protein expression and the effect was partially attenuated by MKP5-siRNA. Downstream of p38 signaling by T+I, NFκB-luc activity and cytokine mRNA accumulation were blocked by curcumin. These results show that MKP5 is up-regulated by curcumin and mediates anti-inflammatory activities of curcumin in normal prostatic E-PZ cells.

In our previous study we found that 1,25D up-regulated MKP5 only in primary E-PZ cells and cells cultured from primary adenocarcinomas of the prostate, but not in the PCa cell lines DU 145, PC-3 and LNCaP (Nonn et al. 2006). 1,25D typically alters gene expression through binding to the vitamin D receptor (VDR). VDR is a classical steroid receptor which translocates to the nucleus and binds to vitamin D response elements (VDREs) in the promoter regions of genes. This is the likely mechanism for regulation of MKP5 by 1,25D because the MKP5 promoter contains a putative VDRE and up-regulation of MKP5 by 1,25D was dependent upon VDR (Nonn et al. 2006). We did not find that curcumin up-regulated any of the classic VDR-regulated genes and this photochemical has not been reported to alter gene expression through

VDR activation. The mechanism for MKP5 up-regulation by curcumin is probably distinct from that of 1,25D, and therefore MKP5 regulation by curcumin was examined in the immortal PCa cell lines DU 145, PC-3, LNCaP and LAPC-4. I found that PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain the ability to up-regulate MKP5 in response to curcumin and that curcumin reduced p38-mediated, but not NFκB-mediated, pro-inflammatory signaling by T+I in DU 145 cells.

Two other phytochemicals, resveratrol and 6-gingerol, have been shown to inhibit p38 phosphorylation and NFκB activation in mouse skin (Kundu et al. 2004; Kim et al. 2005). Since MKP5 was up-regulated by 1,25D and curcumin, by presumably different mechanisms, we wondered if MKP5 could be up-regulated and play a role in the anti-inflammatory activities of resveratrol and 6-gingerol in the prostate. The results showed that MKP5 was a common target of 1,25D and the phytochemicals curcumin, resveratrol and 6-gingerol in normal prostatic E-PZ cells, whereas in the PCa cell lines MKP5 is up-regulated by curcumin, resveratrol and 6-gingerol but not 1,25D.

The materials, methods, results and figures of the phytochemical experiments are detailed in our *in press* manuscript "Chemopreventive anti-inflammatory properties of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells" (Appendix B.)

Conclusions

We have identified MKP5 as a mediator of anti-inflammatory activities in the prostate. MKP5 is up-regulated by vitamin D and chempreventive phytochemicals. Up-regulation of MKP5 inhibited p38 phosphorylation and diminished downstream pro-inflammatory changes. We observed that up-regulation of MKP5 by 1,25D was unique to prostate cells derived from normal tissues or localized adenocarcinomas (Nonn et al. 2006). In the metastases-derived PCa cell lines, MKP5 basal levels were lower and were unchanged by 1,25D treatment (Nonn et al. 2006). Curcumin, in contrast to 1,25D, up-regulated MKP5 in normal prostatic cells as well as in the metastases-derived PCa cell lines DU 145, PC-3, LNCaP and LAPC-4. Although MKP5 is up-regulated by curcumin in the PCa cell lines, its activity is limited to p38-mediated effects on cytokine and COX-2 expression as NFκB activity is p38-independent in those cells.

Given the involvement of MKP5 in anti-inflammatory activities of two chemopreventive compounds, vitamin D and curcumin, we investigated several other phytochemicals suggested to have anti-cancer properties. Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a polyphenol present in grape skin and red wine (Jang et al. 1997). Pezzuto et. al. have demonstrated chemopreventive activity of resveratrol in the initiation, promotion and progression stages of carcinogenesis (Jang et al. 1997). In PCa cell lines, resveratrol inhibits cell growth and decreases androgen receptor signaling (Hsieh and Wu 1999). [6]-gingerol, the major pungent phenolic found in ginger (*Zingiber officinale* Roscoe, Zingiberaceae), has been utilized extensively in oriental medicine for alleviation of inflammation and gastro-intestinal ailments (Newall 1996). However, prostate-specific anti-cancer activities of [6]-gingerol of have not yet been studied. Both resveratrol and [6]-gingerol inhibit phorbol ester-induced COX-2 expression and NFκB activation (Kundu et al. 2004; Kim et al. 2005), activities we have shown can be mediated by MKP5. We found that resveratrol and [6]-gingerol, like vitamin D and curcumin, up-regulated MKP5 in normal prostatic E-PZ cells and in the PCa cell lines, with the exception of resveratrol in the LNCaP cells. In general, we found that LNCaP cells had limited ability to up-regulate MKP5 in response to any of the phytochemicals, suggesting the presence of genomic or epi-genomic suppression. Since MKP5 is a common target of these and potentially other phytochemicals, it is possible that combination dosing strategies could amplify MKP5 up-

regulation, perhaps increasing anti-inflammatory activities while decreasing dosage of individual phytochemicals, thus decreasing side effects.

The fact that the PCa cell lines retain the ability to up-regulate MKP5 in response to phytochemicals is relevant to PCa therapy. It shows that not only can phytochemicals ingested in the diet and via supplements play a role in PCa prevention, but also that phytochemicals can be exploited for use in PCa treatment. P38 MAPK has diverse biological properties and activation can mediate apoptosis or cell survival, depending upon the type of stress, cell background and activities of the other MAPKs (Roux and Blenis 2004). Therefore, whereas inhibition of the p38 pathway in normal tissue is an anti-inflammatory chemopreventive activity, p38 inhibition in PCa could affect cell survival pathways leading to increased sensitivity to chemotherapies. In fact, all of the phytochemicals we analyzed are currently under heavy investigation for their utility in cancer treatment and analogs with enhanced anti-tumor activity are being tested.

In conclusion, these experiments show that 1) MKP5 is a potent inhibitor of pro-inflammatory signaling in prostate cells and 2) MKP5 may be a common mediator of vitamin D and phytochemical anti-inflammatory activities. Our data suggest that, in the prostate, TNFα- and IL-1β-induced inflammatory cell recruitment could theoretically be decreased by phytochemical-induced MKP5, leading to a reduction in prostatic inflammation. Also, in contrast to 1,25D, which does not up-regulate MKP5 in PCa cell lines, curcumin, resveratrol and gingerol were also able to up-regulate MKP5 and inhibit the p38 pathway in the PCa cell lines, implicating potential utility in management of early or advanced PCa. Although we believe MKP5 to be an important mediator of inflammation in prostate cells, we are not down-playing other molecular effects of the phytochemicals. Ultimately, vitamin D and phytochemicals have the ability to affect many pathways and a greater understanding of their mechanisms of action will facilitate exploitation of these naturally occurring “drugs” for cancer prevention and therapy.

Key Accomplishments

Key Research Accomplishments/Significant findings:

1. 1,25D decreased UV and inflammation-stimulated p38 phosphorylation and subsequent cytokine production by increasing MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells.

2. MKP5-mediated p38 inactivation by 1,25D occurs in primary cultures of normal prostatic epithelial cells and not in PCa cell lines.

3. Combination treatment of prostate cells with TNFα and IL-1β stimulates NFkB signaling and pro-inflammatory cytokine production in a p38 dependent manner.

4. Increased expression of MKP5 in prostate cells decreased p38 phosphorylation and reduced NFkB activation and pro-inflammatory cytokine production.

5. Curcumin up-regulated MKP5 and subsequently decreased p38 phosphorylation and reduced NFkB activation and pro-inflammatory cytokine production in normal prostate cells and DU 145 prostate cancer cells.

6. The phytochemicals resveratrol and gingerol also up-regulate MKP5 mRNA in both normal and malignant prostate cells.

7. In summary, our findings show direct anti-inflammatory activity of MKP5 in prostate cells and suggest that up-regulation of MKP5 by phytochemicals and vitamin D may contribute to their chemopreventive actions by decreasing prostatic inflammation.

Key Training Accomplishments:

- Faculty appointment in the Department of Pathology, University of Illinois at Chicago

Reportable Outcomes

- Primary Author Manuscripts:

  Nonn L, Duong DT, Peehl DM. Chemopreventive anti-inflammatory properties of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. *Carcinogenesis* in press, 2006.

  Nonn L, Peng LH, Feldman D, Peehl DM. Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D. *Cancer Res* 66(8):4516-24, 2006.

- Contributing Author Manuscripts:

  Krishnan AV, Moreno J, Nonn L, Malloy P, Swami S, Peng L, Peehl DM, Feldman D. Novel pathways that contribute to the anti-proliferative and chemopreventive activities of calcitriol in prostate cancer. *J Steroid Biochem Mol Biol* in press, 2006.

  Husbeck B, Nonn L, Peehl DM, Knox SJ. Tumor-selective killing by selenite in patient-matched pairs of normal and malignant prostate cells. *Prostate* 66(2): 218-225, 2006.

  Moreno J, Krishan AV, Swami S**, Nonn L**, Peehl DM, Feldman D. Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res* 65(17):7917-7925, 2005.

  Ma J, **Nonn L**, Campbell M, Hewison M, Feldman D, Peehl D. Mechanisms of decreased vitamin D 1-alpha-hydroxylase activity in prostate cancer cells. *Mol Cell Endocrinology,* 221(1-2): 67-74, 2004.

  Peehl DM, Shighal R, **Nonn L**, Seto E, Krishnan AV, Brooks JD, Feldman D. Molecular activity of 1,25-dihydroxyvitamin D3 in primary cultures of human prostatic epithelial cells revealed by cDNA microarray analysis. *J Steroid Biochem Mol Bio* 92(3):131-41, 2004.

- Awards and Meetings:

  April 2005, Attended and presented at Keystone meeting on cancer prevention.

November 2004, Attended American Association for Cancer Research meeting “Basic, Translational, and Clinical Advances in Prostate Cancer” and received Scholar-in-training travel supplement award

- Funding:

  The results of this DOD-funded research will be the basis for my future grant applications as a principle investigator. I plan to submit applications for the DOD-PCa New Investigator Award (April 2007) and for a K Award (Feb 2007).

- Career:

  September 2006, Faculty appointment:
  Research Assistant Professor, University of Illinois at Chicago Pathology Department

Appendices

(A) **Nonn L**, Duong DT, Peehl DM. Chemopreventive anti-inflammatory properties of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. *Carcinogenesis* in press, 2006.
(B) **Nonn L**, Peng LH, Feldman, D, Peehl DM. Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D. *Cancer Res* 66(8):4516-24, 2006.
(C) Curriculum Vitae

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Inhibition of p38 by Vitamin D Reduces Interleukin-6 Production in Normal Prostate Cells via Mitogen-Activated Protein Kinase Phosphatase 5: Implications for Prostate Cancer Prevention by Vitamin D

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Abstract

Although numerous studies have implicated vitamin D in preventing prostate cancer, the underlying mechanism(s) remains unclear. Using normal human prostatic epithelial cells, we examined the role of mitogen-activated protein kinase phosphatase 5 (MKP5) in mediating cancer preventive activities of vitamin D. Up-regulation of MKP5 mRNA by 1,25-dihydroxyvitamin-D_3 (1,25D) was dependent on the vitamin D receptor. We also identified a putative positive vitamin D response element within the MKP5 promoter that associated with the vitamin D receptor following 1,25D treatment. MKP5 dephosphorylates/inactivates the stress-activated protein kinase p38. Treatment of prostate cells with 1,25D inhibited p38 phosphorylation, and MKP5 small interfering RNA blocked this effect. Activation of p38 and downstream production of interleukin 6 (IL-6) are proinflammatory. Inflammation and IL-6 overexpression have been implicated in the initiation and progression of prostate cancer. 1,25D pretreatment inhibited both UV- and tumor necrosis factor α–stimulated IL-6 production in normal cells via p38 inhibition. Consistent with inhibition of p38, 1,25D decreased UV-stimulated IL-6 mRNA stabilization. The ability of 1,25D to up-regulate MKP5 was maintained in primary prostatic adenocarcinoma cells but was absent in metastases-derived prostate cancer cell lines. The inability of 1,25D to regulate MKP5 in the metastasis-derived cancer cells suggests there may be selective pressure to eliminate key tumor suppressor functions of vitamin D during cancer progression. These studies reveal MKP5 as a mediator of p38 inactivation and decreased IL-6 expression by 1,25D in primary prostatic cultures of normal and adenocarcinoma cells, implicating decreased prostatic inflammation as a potential mechanism for prostate cancer prevention by 1,25D. (Cancer Res 2006; 66(8): 4516-24)

Introduction

Prostate cancer (PCa) is unique among malignancies in that it generally grows very slowly, likely for decades, before symptoms arise and a diagnosis is finally made. Seemingly, the latency observed in PCa should provide a long window of opportunity for intervention by chemopreventive agents. Laboratory and epidemiologic studies have shown a potential role for vitamin D in the prevention of PCa. Evidence supporting a role for vitamin D in PCa prevention began with studies that linked reduced serum levels of vitamin D metabolites to PCa incidence. Low serum levels of 1,25-dihydroxyvitamin D_3 (1,25D), the active vitamin D metabolite, are associated with increased risk of PCa in older men (1). Decreased serum levels of 25-hydroxyvitamin D_3 (25D), the circulating precursor to 1,25D, also correlate with an increased risk of PCa (1). The latter finding has become more compelling since the discovery that prostate cells are not only sensitive to circulating 1,25D but can also synthesize 1,25D from circulating 25D. Conversion of 25D to active 1,25D by vitamin D 1α-hydroxylase (1) occurs within the normal prostate and suggests that local production of 1,25D may play a critical role in maintaining normal growth and differentiation.

Studies showing that 1,25D inhibits the growth of primary cultures of prostate cells, established PCa cell lines, and prostate xenograft tumors provide direct evidence for anticancer activity of vitamin D (1). However, mechanisms other than growth inhibition may be responsible for the prevention of PCa by vitamin D.

Using cDNA microarrays, we recently identified a new vitamin D–responsive gene, mitogen-activated protein kinase phosphatase 5 (*MKP5*; ref. 2). The up-regulation of MKP5, also known as dual-specificity phosphatase 10, was consistently increased by 1,25D treatment of primary cultures of prostatic epithelial cells (2). As a member of the dual-specificity MKP family of proteins that dephosphorylate mitogen-activated protein kinases, MKP5 dephosphorylates p38 and c-jun NH_2-terminal kinase, but not extracellular signal–regulated kinase (3, 4).

The potential ability of vitamin D to inhibit p38 through MKP5 is of interest to PCa prevention because p38 is activated by oxidative stress, hypoxia, and inflammation (5), all of which contribute to PCa development (6, 7). In particular, inflammation plays a causal role in the progression of many cancers including liver, bladder, and gastric cancers (8) and a similar role for inflammation in the development of PCa is now emerging (7). The strongest evidence linking inflammation to PCa is from recent findings that show (*a*) regular administration of nonsteroidal anti-inflammatory drugs significantly decreases PCa risk in older men by 60 to 80% (9, 10) and (*b*) men with chronic and/or acute inflammation of the prostate have an increased risk of developing PCa (7, 11).

Inhibitors of p38 are classically anti-inflammatory, suggesting that some of the activities attributed to vitamin D, including PCa prevention, may be a result of p38 inhibition and decreased inflammation. One of the downstream consequences of p38 protein kinase pathway activation is an increase in proinflammatory cytokine production to amplify the inflammatory response (12, 13). Interleukin 6 (IL-6) is a p38-regulated pleiotropic cytokine that has

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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been historically associated with PCa (14, 15). Elevated levels of IL-6 are found in the serum of PCa patients and primary PCa tumors overexpress IL-6 (14, 16). IL-6 is also involved in the progression of PCa to androgen-independent PCa because it can facilitate androgen receptor signaling in the absence of androgens (15, 17).

Because there is increasing evidence linking inflammation to PCa development, our studies focused on characterizing regulation of IL-6 production via p38 inhibition by 1,25D as a potentially significant cancer prevention activity. Using primary epithelial cell cultures derived from the normal peripheral zone (E-PZ; the major site of origin of prostatic adenocarcinomas; ref. 18) and from primary adenocarcinomas of the prostate (E-CA) as our model system, we characterized MKP5 induction by 1,25D and revealed MKP5 as the mediator of p38 kinase inhibition and decreased IL-6 production by 1,25D. Prostate cancer cell lines derived from metastases, however, have lost the ability to up-regulate MKP5 in response to 1,25D, suggesting selection against this anticancer activity of vitamin D.

Materials and Methods

Cell culture and reagents. Human primary prostatic epithelial and stromal cells were derived from radical prostatectomy specimens. The patients did not have prior chemical, hormonal, or radiation therapy. Histologic characterization and cell culture of the prostate cells was as previously described (19). Epithelial cells (E-PZ and E-CA) were cultured in supplemented MCDB 105 (Sigma-Aldrich, St. Louis, MO) or PFMR-4A as previously described (19). Stromal cells (F-PZ) were cultured in MCDB 105/10% fetal bovine serum (FBS). pRNS1-1 cells are immortalized E-PZ cells (20) and are cultured in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA). Human PCa cell lines LNCaP, PC-3, and DU 145 were acquired from American Type Culture Collection (Manassas, VA). LNCaP cells were cultured in MCDB 105/10% FBS and PC-3 and DU 145 in DMEM (Invitrogen)/10% FBS. Normal human keratinocytes were obtained from Cambrex (East Rutherford, NJ) and cultured according to the instructions of the supplier. Squamous cell carcinoma cell lines SCC-25 and A431 were obtained from Dr. Paul Khavari (Stanford University, Stanford, CA) and cultured in DMEM/10% FBS. All chemicals were obtained from Sigma-Aldrich unless otherwise noted. 1,25-dihydroxyvitamin D_3 (Biomol International, Plymouth Meeting, PA) was reconstituted in 100% ethanol at 10 mmol/L and stored at −20°C.

RNA isolation and quantitative real-time reverse transcription-PCR. RNA was isolated from cells by Trizol (Invitrogen) followed by chloroform extraction. The aqueous phase was then precipitated in 100% isopropanol and the pellet washed in 75% ethanol before resuspension in water. RNA concentration and quality were determined by absorbance ratio at 260/280 nm using a UV spectrophotometer. Total RNA (2 μg) was reverse transcribed using Thermoscript RT (Invitrogen). Resulting cDNA was used for quantitative PCR amplification with gene-specific primers and the DyNAMO SYBR Green kit (Finnzymes, Espoo, Finland) in the Opticon 2 thermocycler (MJ Research, South San Francisco, CA). PCR conditions for all primer sets were optimized and have similar amplification efficiency under the following thermocycler conditions: 95°C 5 minutes, 34 × (95°C 30 seconds, 58°C 30 seconds, 72°C 60 seconds), 72°C 5 minutes. Relative mRNA levels were calculated from the point where each curve crossed the threshold line (Ct) using the following equation: Rel. value = $2^{-[Ct(control) - Ct(test)]test\ gene} / 2^{-[Ct(control) - Ct(test)]housekeeping\ gene}$ (21). Reactions were done in triplicate and the values normalized to the expression of the housekeeping gene TATA-box binding protein (*TBP*; ref. 22). Primer sets were TBP, 5′-tgctgagaagagtgtgctggag-3′ and 5′-tctgaataggctgtggggtc-3′; total MKP5, 5′-atcttgcccttcctgttcct-3′ and 5′-attggtcgtttgcctttgac-3′; MKP5 isoform 1 specific, 5′-tgaatgtgcgagtccatagc-3′ and 5′-gttagcagggcaggtggtag-3′; MKP5 isoform 2 specific, 5′-tggatgcagctgagattctg-3′ and 5′-ggttctgcttgttgctgtca-3′; MKP5 isoform 3 specific, 5′-atttatgaagtggacttagt-3′ and 5′-ggttctgcttgttgctgtca-3′; and CYP24, 5′-ggcaacagttctgggtgaat-3′ and 5′-tatttgcggacaatccaaca-3′.

Cell lysate preparation and immunoblot. Cells were lysed in ice-cold 1× Cell Lysis Buffer (Cell Signaling, Beverly, MA) containing 1 μmol/L phenylmethylsulfonyl fluoride and 100 nmol/L okadaic acid. Cells were disrupted by sonication and insoluble cell debris removed by centrifugation at 15,000 × *g*, 4° C. Protein concentrations of the cell lysates were quantified using the Bio-Rad Protein Dye (Bio-Rad, Hercules, CA). Cell lysates were used fresh or stored at −70°C. Cell lysates (10-30 μg) were mixed with LDS NuPAGE Sample Buffer and separated by electrophoresis through 10% NuPAGE Bis-Tris Gels (Invitrogen) and transferred onto polyvinylidene difluoride membrane. Fresh cell lysates were used for analysis of phosphorylated proteins. Membranes were probed with the following primary antibodies: anti–phospho-p38 rabbit polyclonal and anti-p38 rabbit polyclonal from Cell Signaling, anti–vitamin D receptor (VDR) monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti-actin (Santa Cruz Biotechnology). Following primary antibody incubation overnight at 4°C, the blots were incubated with appropriate secondary horseradish peroxidase–conjugated antibodies (Cell Signaling) and developed with HyGlo enhanced chemiluminescence reagent (Denville Scientific, Metuchen, NJ).

Small interfering RNA transfection. Cells at 75% confluency were transfected with 10 nmol/L of negative control (Ambion, Austin, TX), VDR-specific small interfering RNA (siRNA; Santa Cruz Biotechnology), or MKP5-specific siRNA (Ambion) using siPORT NeoFX (Ambion). Cells were used for experiments after transfection as indicated in results and figure legends.

Chromatin immunoprecipitation. Chromatin immunoprecipitation assays were carried out using Upstate Biologics (Waltham, MA) protocol and reagents. Briefly, cells (1-100-mm dish per treatment) were cross-linked with 1% formaldehyde, harvested, and sonicated before immunoprecipitation. One microgram each of anti-VDR (H-81) and anti-VDR (N-20) rabbit polyclonal antibodies (Santa Cruz Biotechnology) was used for overnight 4°C immunoprecipitation. Protein A-agarose beads were then used to pull down immune complexes. Beads were washed, then reverse cross-linked with NaCl at 65°C. The DNA was extracted with spin columns. PCR (30 cycles) was done on 10% of the recovered DNA using primers flanking the vitamin D response element (VDRE) in the MKP5 promoter; VDRE-MKP5: 5′-ccagagccgagtgcaaatag-3′ and 5′-gcaactttcctgcagttcc-3′. Primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (5′-cggctactagcggttttacg-3′ and 5′-aagaagatgcggctgactgt-3′) were used as a negative control. PCR products were electrophoresed through 1.5% agarose gels containing 1 μg/mL of ethidium bromide and visualized by UV.

UV irradiation. Prostate cell cultures were exposed to 1,000 J/m^2 (~45 seconds) of UVB irradiation using calibrated UVB bulbs with a Kodaceal filter. The lid of the cell culture dish and phenol red–containing media were removed during UV irradiation.

***In vitro* p38 kinase assay.** All reagents, antibodies, and protocol for this assay were supplied by Cell Signaling Technologies. Fresh cell lysate was prepared as described above under *Cell lysate preparation and immunoblot.* Cell lysates containing 250 μg of protein were incubated overnight with immobilized phospho-p38 monoclonal antibody to immunoprecipitate activated p38. Bead-immune complexes were washed and resuspended in 1× kinase buffer containing 200 μmol/L ATP and recombinant activating transcription factor-2 (ATF-2) fusion protein as the substrate and incubated at 30°C for 30 minutes to allow phosphorylation of ATF-2. Reactions were terminated by addition of LDS sample buffer. Samples were then heated at 95°C for 5 minutes and kinase activity was determined by immunoblot analysis with phospho-ATF-2 antibody. Input protein (20 μg) was also immunoblotted and probed with anti-p38.

IL-6 ELISA. Prostate cells (10^5) were plated in 24-well culture plates. After 24 hours, fresh media containing various agents were added (as described in Results and figure legends). Conditioned media were collected following treatment and used at a 2:1 dilution to determine amount of secreted IL-6 with the Human IL-6 ELISA Kit II (BD Biosciences, San Diego, CA). Results were calculated from a standard curve and are expressed as pg/mL IL-6/10^6 cells or pg/10^5 cells.

IL-6 promoter activity. A pGL3 luciferase construct containing a 651-bp fragment of the IL-6 promoter and pRL-null-renilla (Promega, Madison, WI) were transiently transfected into E-PZ cells using NeoFX reagent. pGL3-IL-6 was a generous gift from Dr. Oliver Eikelberg at the University of Giessen (Giessen, Germany). Eight hours after transfection, cells were treated with vehicle or 50 nmol/L 1,25D. Cells were UV irradiated 14 hours after vehicle or 1,25D treatment. Luciferase activity was measured 24 hours after UV using the Dual-Luciferase Assay Kit (Promega). The ratio of luciferase to renilla-luciferase was determined to correct for transfection efficiency.

Results

1,25D increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells. We previously showed that MKP5 mRNA was increased 3- to 10-fold after 6 hours of treatment with 50 nmol/L of 1,25D in three independent primary cultures of normal human prostatic epithelial cells (E-PZ; ref. 2). The optimal concentration of 1,25D required to up-regulate MKP5 mRNA was determined by quantitative reverse transcription-PCR (RT-PCR). A dose-response curve showed that 1 nmol/L of 1,25D was sufficient to increase MKP5 mRNA in E-PZ cells by 6 hours, but 50 nmol/L of 1,25D was required to maintain MKP5 mRNA up-regulation at 24 hours (Fig. 1*A*). Higher concentrations of 1,25D are needed at the time points >12 hours because high density E-PZ cells rapidly metabolize and inactivate 1,25D (23). In all subsequent experiments, 50 nmol/L of 1,25D was used to treat subconfluent cultures of E-PZ cells. Upon treatment with 50 nmol/L of 1,25D, increased MKP5 expression was observed as early as 3 hours and maintained for 24 hours in E-PZ cells (Fig. 1*B*).

The *MKP5* gene is located on chromosome 1 and is transcribed into three distinct mRNA splice variants that putatively encode two different proteins (24). MKP5 splice variant 1 encodes the full-length 52-kDa protein whereas variants 2 and 3 both encode a truncated 16-kDa protein that only contains the dual-specificity phosphatase domain (Fig. 1*D*). Because the biological significance of the MKP5 splice variants has not yet been characterized, we examined the ability of 1,25D to regulate the mRNA expression of the three MKP5 splice variants by quantitative RT-PCR. All of the MKP5 mRNA splice variants were induced following 6 hours of treatment with 50 nmol/L of 1,25D, but splice variant 1 seemed to be most abundant and achieved the highest level of mRNA induction in the E-PZ cells (Fig. 1*C*). Primers within exons 3 to 5 of MKP5, the conserved region present in all MKP5 mRNAs, were used for all subsequent experiments.

Up-regulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR on 1,25D treatment. The rapid induction of MKP5 mRNA by 1,25D suggested that MKP5 is a direct target of 1,25D. Direct targets of 1,25D contain one or more VDREs in the promoter region, which mediate transcriptional regulation by VDR binding. Knockdown of VDR levels by VDR siRNA in E-PZ cells abolished the induction of MKP5 by 1,25D (Fig. 2*A*), showing that induction of MKP5 by 1,25D is VDR dependent. The mRNA expression of vitamin D 24-hydroxylase (CYP24), a well-characterized target of 1,25D, was similarly suppressed in cells transfected with VDR siRNA (Fig. 2*A*) whereas the expression of *TBP*, a housekeeping gene, was not affected by VDR siRNA transfection. On sequence analysis, a putative VDRE was identified −1,320 bp upstream of the 5′ untranslated region in the MKP5 promoter (Fig. 2*B*). The putative MKP5-VDRE was highly similar to the characterized VDREs present in the promoters of CYP24 and parathyroid hormone–related protein. Chromatin immunoprecipitation assay showed an increased interaction between VDR and the putative MKP5-VDRE that exhibited time-dependent changes on stimulation with 50 nmol/L of 1,25D in E-PZ cells (Fig. 2*C*). The cyclic nature of VDR interaction with the MKP5 promoter observed in the chromatin immunoprecipitation experiments is consistent with previously described interactions of nuclear hormone receptors with DNA (25). The siRNA and chromatin immunoprecipitation data together provide strong support that MKP5 is directly regulated by 1,25D at the transcription level.

MKP5-mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines. Phosphorylation of p38 is required for activation of p38 kinase activity. MKP5 dephosphorylates p38, thus reducing p38 kinase activity. Vitamin D has been shown to

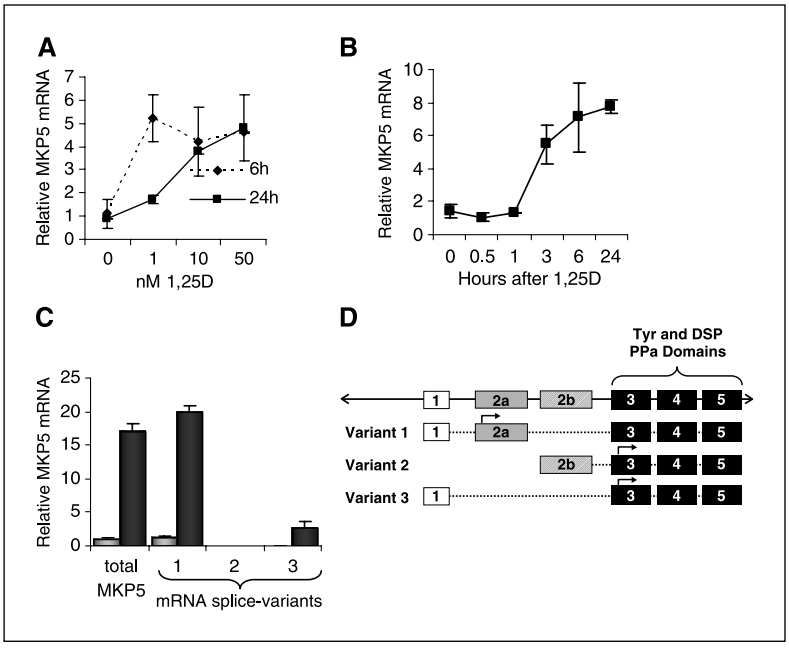

Figure 1. 1,25D increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells (E-PZ). Quantitative RT-PCR measurement of MKP5 mRNA levels in E-PZ cells, 6 hours (*dashed line*) and 24 hours (*solid line*) after treatment with 1, 10, and 50 nmol/L 1,25D (*A*) and 0.5, 1, 3, 6 and 24 hours following 50 nmol/L 1,25D treatment (*B*). *C,* quantitative RT-PCR analysis of MKP5 mRNA splice variants in E-PZ cells after 6-hour vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*) treatment (mRNA levels for isoform 2 treated with vehicle and 1,25D are 0.003 ± 0.0007 and 0.014 ± 0.0004, respectively, but are not visible on this graph). *D,* diagram of MKP5 mRNA splice variants. Quantitative RT-PCR results are shown relative to untreated control and normalized to expression of housekeeping gene *TBP*. Each experiment was run in triplicate and graphs are representative of two or more separate experiments with different patient-derived E-PZ cells. *Bars,* SD.

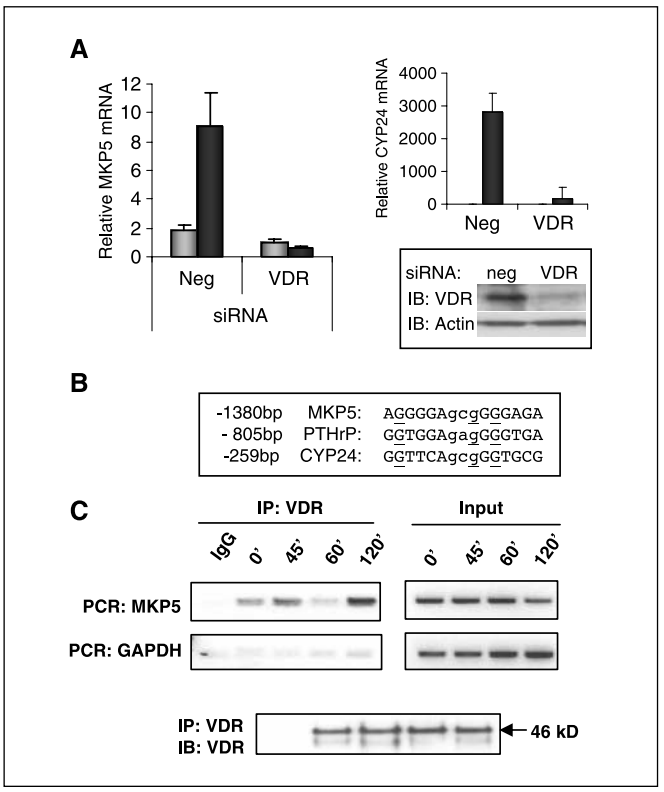

Figure 2. Up-regulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR on 1,25D treatment. *A,* quantitative RT-PCR analysis of MKP5 mRNA 6 hours after treatment with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*) in E-PZ cells that were transfected for 24 hours with negative control siRNA or VDR siRNA. CYP24 gene expression was included as positive control for VDR knockdown (untreated mRNA level of CYP24 is equal to one and is not visible on this graph) and immunoblot of nuclear lysate (10 μg) verified VDR knockdown by the siRNA. *B,* diagram of putative VDRE located −1,380 bp of 5′UTR in MKP5 promoter aligned with validated VDREs in parathyroid hormone–related protein and CYP24. *C,* chromatin immunoprecipitation analysis and PCR of putative VDRE in MKP5 promoter and GAPDH promoter following 50 nmol/L 1,25D treatment using rabbit polyclonal VDR antibody for pulldown. Immunoblot of protein precipitate probed with mouse monoclonal VDR antibody shows specific pulldown of VDR. Results are representative of four or more separate experiments with different patient-derived E-PZ cells. Quantitative RT-PCR results are shown relative to untreated control and normalized to expression of housekeeping gene *TBP*. Each experiment was run in triplicate and graphs were representative of two or more separate experiments. *Bars,* SD.

inhibit osmotic stress–stimulated p38 phosphorylation in keratinocytes (26). We observed a similar inhibition of p38 phosphorylation by 1,25D in E-PZ cells (Fig. 3*A*). E-PZ cells were pretreated with 1,25D for 14 hours to allow for sufficient up-regulation of MKP5 protein. MKP5 protein levels could not be directly monitored in this study due to lack of an appropriate antibody. After pretreatment with 1,25D, osmotic stress–stimulated phosphorylated p38 levels were appreciably decreased compared with levels in cells not treated with 1,25D. Transfection with MKP5-specific siRNA attenuated induction of MKP5 mRNA by 1,25D compared with negative control siRNA (Fig. 3*B*) and abolished the suppression of p38 phosphorylation by 1,25D (Fig. 3*A*), implicating MKP5 as the mediator of p38 inactivation by 1,25D.

We examined the effect of 1,25D on MKP5 in various other prostate-derived cells in comparison with the primary cultures of E-PZ cells. The results showed that, like E-PZ cells, pRNS-1-1 cells also induce MKP5 (Fig. 3*C*). In contrast, prostate stromal cells (F-PZ) and established PCa cell lines (PC-3, LNCaP, and DU 145) did not up-regulate MKP5 mRNA following 1,25D treatment (Fig. 3*C*). pRNS-1-1 cells were generated by SV40 transformation and immortalization of E-PZ cells and are not growth inhibited by 1,25D, although they retain VDR and other responses to 1,25D (1). Prostatic stromal cells too express VDR and show certain responses to 1,25D despite lack of induction of MKP-5 in these cells by 1,25D (1). The PCa cell lines PC-3, LNCaP, and DU 145 all express VDR and respond to 1,25D in other ways (1). Immunoblot analysis showed that in DU 145, PC-3, and LNCaP cells, 1,25D pretreatment did not alter NaCl-induced p38 phosphorylation (Fig. 3*D*). These data suggest that MKP5 may specifically mediate 1,25D activity in normal prostate cells and that this activity is lost in advanced PCa.

We suspect that MKP5 up-regulation by 1,25D is not unique to prostatic epithelium. Because inactivation of p38 by 1,25D was reported in keratinocytes, we examined the expression of MKP5 in these cells. Similarly to prostatic epithelial cells, normal human keratinocytes showed up-regulation of MKP5 mRNA on treatment with 1,25D, suggesting that MKP5 may mediate p38 inactivation in keratinocytes as well (Supplementary Fig. S1*A*). Similarly to the PCa cell lines, up-regulation of MKP5 by 1,25D was attenuated in the human squamous cell carcinoma cell lines A431 and SCC-25 (Supplementary Fig. S1*B*).

MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D. Published studies have shown that 1,25D inhibits UV-induced IL-6 production in keratinocytes; however, no mechanism has been proposed (27). IL-6 overexpression has been strongly associated with PCa progression and, therefore, inhibition of IL-6 may play an important role in PCa prevention. Because IL-6 induction is downstream of p38 activation and often dependent on p38 activation (13, 28), we tested the role of MKP5 in regulating IL-6 expression in E-PZ cells. An *in vitro* p38 kinase activity assay, using ATF-2 as the substrate, showed that 1,25D pretreatment decreased basal and UV-stimulated p38 activity in E-PZ cells (Fig. 4*A*). The 1,000 J/m^2 dose of UVB irradiation used in these experiments did not induce apoptosis or necrosis in E-PZ cells (data not shown). Consistent with p38 inactivation, secreted levels of IL-6 protein following UV treatment were suppressed by 1,25D pretreatment (Fig. 4*B*). SB202190, a specific p38 inhibitor, similarly decreased UV-stimulated IL-6 production (Fig. 4*B*). A decrease in IL-6 mRNA expression was observed in 1,25D pretreated samples and this decrease was blocked by transient transfection with MKP5 siRNA (Fig. 4*C*). The primary mechanism for increased IL-6 production following p38 activation is through IL-6 mRNA stabilization rather than increased mRNA transcription (29). To determine if this is also true for 1,25D regulation of IL-6 mRNA, we examined the effect of 1,25D on IL-6 promoter activity in the absence or presence of UV irradiation. We found that neither UV irradiation nor 1,25D treatment significantly altered IL-6 promoter activity as determined by luciferase assay in E-PZ cells (Fig. 4*D1*), suggesting that 1,25D is not altering mRNA transcription. When 1 μmol/L of actinomycin D was used to inhibit new mRNA transcription, we observed that UV irradiation caused IL-6 mRNA stabilization and 1,25D pretreatment decreased the UV-induced IL-6 mRNA stabilization (Fig. 4*D2*). Under basal conditions, IL-6 mRNA half-life was <45 minutes in E-PZ cells. In UV-irradiated cells, IL-6 mRNA half-life increased to >90 minutes whereas UV irradiation did not significantly alter IL-6 mRNA half-life in 1,25D pretreated cells (Fig. 4*D2*).

1,25D inhibits tumor necrosis factor α–stimulated p38 activation and IL-6 production. The ability of 1,25D to inhibit

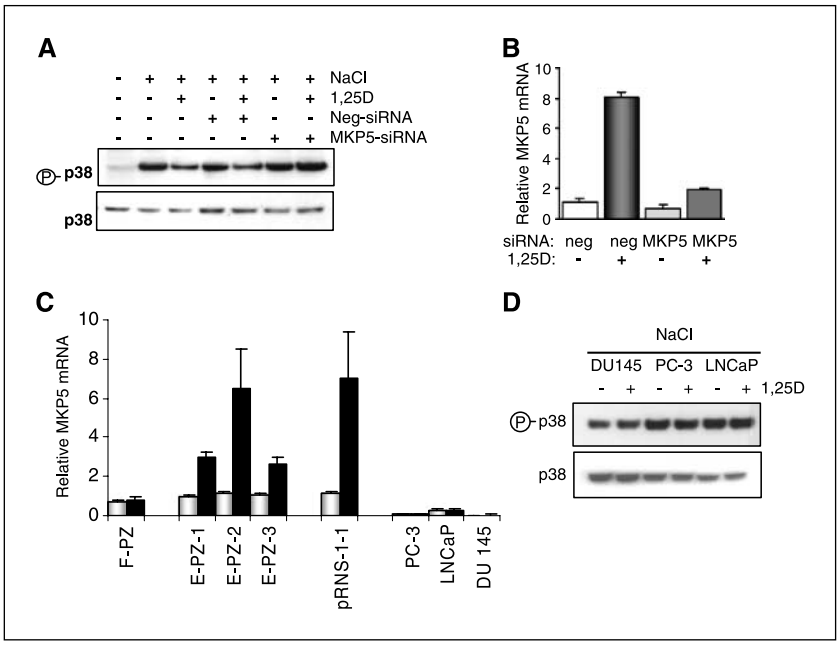

Figure 3. MKP5-mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines. *A,* immunoblot analysis of p38 phosphorylation in 20 μg of E-PZ cell lysate 20 minutes after treatment with 0.5 mol/L NaCl. Before NaCl treatment, E-PZ cells were transfected with negative control or MKP5 siRNA for 4 hours, then treated with vehicle or 50 nmol/L 1,25D for 14 hours. *B,* quantitative RT-PCR analysis of MKP5 mRNA expression in E-PZ cells transfected with negative control siRNA (*open columns*) or MKP5 siRNA (*striped columns*) 4 hours before vehicle (*light columns*) or 1,25D (*shaded columns*) treatment for 12 hours. Representative of at least three separate experiments with different patient-derived E-PZ cells. Results are displayed relative to negative siRNA control and normalized to expression of the housekeeping gene *TBP. Bars,* SD of triplicate samples. *C,* quantitative RT-PCR analysis of MKP5 mRNA 6 hours after treatment with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*) in primary cultures of prostate stroma (F-PZ) in three different cultures of normal primary prostatic epithelial cells (derived from normal peripheral zone designated E-PZ) and in prostate cell lines pRNS-1-1, LNCaP, PC-3, and DU 145. Quantitative RT-PCR results are displayed relative to control and normalized to expression of the housekeeping gene *TBP. Bars,* SD. *D,* immunoblot analysis of p38 phosphorylation 20 minutes after treatment with 0.5 mol/L NaCl in DU 145, PC-3, and LNCaP cells pretreated for 14 hours with vehicle or 50 nmol/L 1,25D.

p38 phosphorylation was further investigated using a more physiologic stress, tumor necrosis factor α (TNF-α). TNF-α is a proinflammatory cytokine released by inflammatory cells that can trigger cell proliferation, necrosis, apoptosis, and induction of other cytokines (12). Interestingly, elevated serum levels of TNF-α are associated with aggressive pathology and decreased survival of PCa patients (16). In E-PZ cells, TNF-α does not induce apoptosis but does significantly slow cell growth (30). TNF-α binds cell-surface receptors which signal through multiple pathways, including p38 kinase, to increase production of IL-6 and other cytokines (12). Immunoblot analysis showed that TNF-α-stimulated p38 phosphorylation was attenuated by 1,25D pretreatment, similar to the

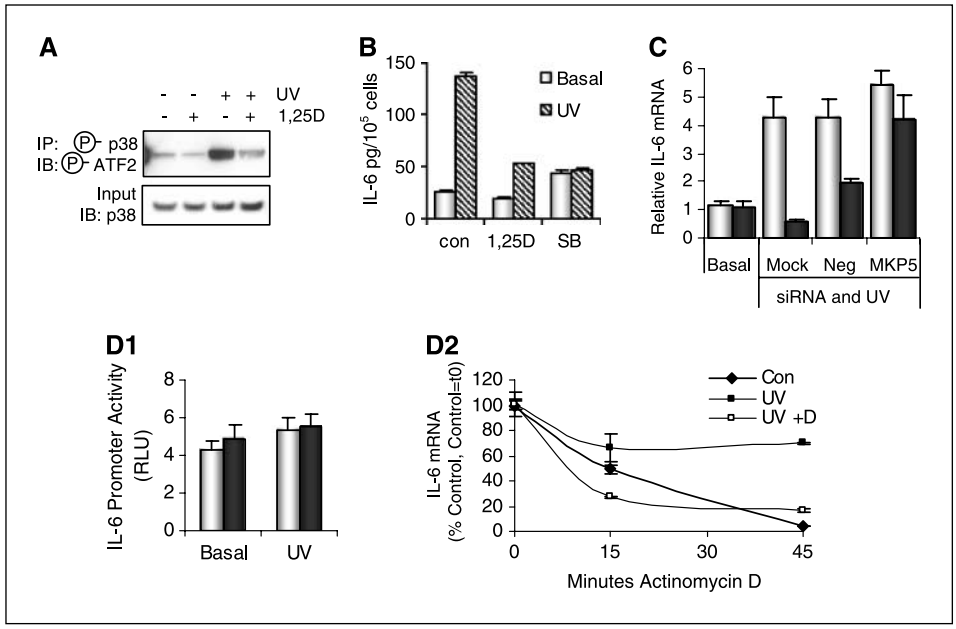

Figure 4. MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D. *A, in vitro* p38 kinase activity assay, using ATF-2 as a substrate, in E-PZ cells 20 minutes after 1,000 J/m^2 UVB irradiation in E-PZ cells pretreated 14 hours with vehicle or 50 nmol/L 1,25D. *B,* ELISA measurement of secreted IL-6 in cell culture media 24 hours after UVB irradiation (*hatched columns*) in E-PZ cells pretreated with vehicle, 50 nmol/L 1,25D for 14 hours, or 1 μmol/L SB202190 for 1 hour. *C,* quantitative RT-PCR measurement of IL-6 mRNA in E-PZ cells 24 hours after UV irradiation. Cells were either transiently transfected with negative siRNA or MKP5 siRNA for 4 hours, then pretreated for 14 hours with either vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*) before UV. *D1,* luciferase activity of pGL3-IL-6 24 hours after UV irradiation in E-PZ cells pretreated for 14 hours with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*). *D2,* quantitative RT-PCR analysis of IL-6 mRNA stability in E-PZ cells following UV irradiation. Cells were treated with 1 μmol/L actinomycin D for 0, 15, and 45 minutes. Actinomycin was dosed 30 minutes after UV and cells were pretreated 14 hours before UV with vehicle (■) or 50 nmol/L 1,25D (□). IL-6 mRNA levels under basal conditions after 15 to 45 minutes of treatment with 1 μmol/L actinomycin D were shown as control (♦).

effect of 1,25D on UV-stimulated p38 phosphorylation in E-PZ cells (Fig. 5*A*). Changes in IL-6 mRNA and secreted protein levels were followed over a time course after TNF-α stimulation and showed that 1,25D pretreatment attenuated the initial production of IL-6 and completely inhibited the accumulation of IL-6 in the media (Fig. 5*B* and *C*).

1,25D up-regulates MKP5 mRNA and inhibits TNF-α-stimulated IL-6 production in matched pairs of normal and tumor cells from individual PCa patients. Because we observed a difference in regulation of MKP5 by 1,25D between E-PZ cells and PCa cell lines, we analyzed the effect of 1,25D on MKP5 and IL-6 in matched pairs of normal (E-PZ) and tumor (E-CA) cells from two PCa patients with localized Gleason grade 4/3 cancers (individuals *A* and *B*). Quantitative RT-PCR analysis showed that MKP5 mRNA was up-regulated by 1,25D in E-CA as well as E-PZ cells from both individuals (Fig. 6*A*). In addition, IL-6 mRNA and protein levels, following TNF-α stimulation, were decreased by 1,25D pretreatment in all of the matched pairs of normal and cancer cultures (Fig. 6*B* and *C*). Although patient-to-patient variability was evident in the extent of TNF-α-stimulated IL-6 production, cancer cells from primary adenocarcinomas retain the ability to up-regulate MKP5 in response to 1,25D, in contrast to the established metastases-derived cell lines (DU 145, LNCaP, and PC-3).

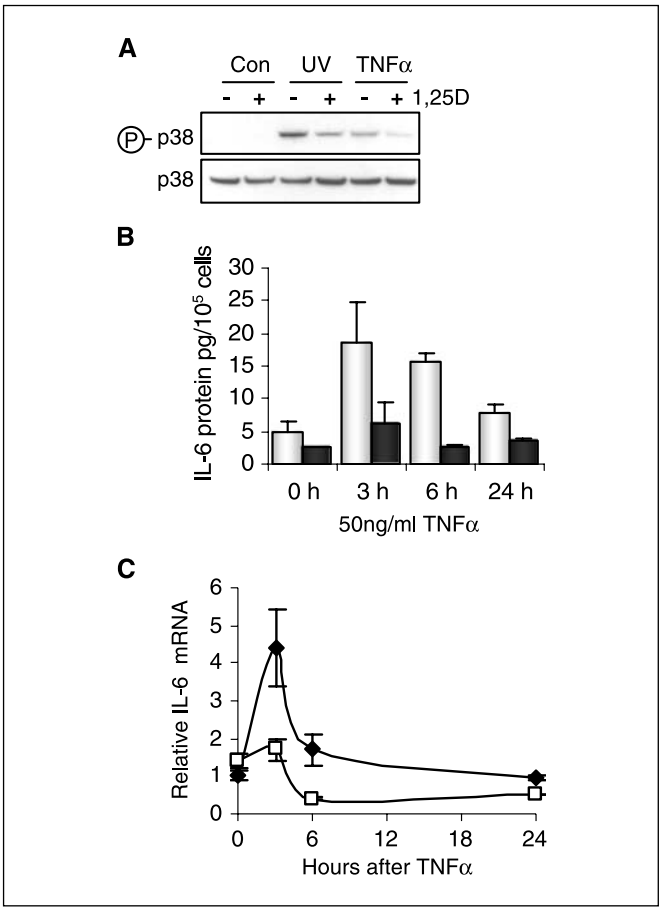

Figure 5. 1,25D inhibits TNF-α-stimulated p38 phosphorylation and IL-6 production. *A,* immunoblot analysis of p38 phosphorylation 20 minutes after treatment with 1,000 J/m^2 UVB or 50 ng/mL TNF-α in E-PZ cells pretreated 14 hours with vehicle or 50 nmol/L 1,25D. *B,* ELISA measurement of secreted IL-6 in cell culture media 3, 6, and 24 hours after 50 ng/mL TNF-α in E-PZ cells pretreated 14 hours with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*). *C,* quantitative RT-PCR analysis of IL-6 mRNA 3, 6, and 24 hours after 50 ng/mL TNF-α in E-PZ cells pretreated for 24 hours with vehicle (♦) or 50 nmol/L 1,25D (□). *IL-6* gene expression relative to untreated control and normalized to expression of the housekeeping gene *TBP. Bars,* SD of triplicate samples.

Discussion

The purpose of these studies was to explore the potential significance of MKP5 in mediating PCa prevention by vitamin D. We previously identified MKP5 as a target of 1,25D in normal human prostatic epithelial cells by microarray analysis (2). The results of our studies suggest that the ability of vitamin D to inhibit p38 signaling, via MKP5 up-regulation, may be a significant antitumor activity of vitamin D (Fig. 7).

MKP5 is likely a direct target of 1,25D, regulated by a positive VDRE in the promoter region of the gene. Treatment of E-PZ cells with 1,25D produced a time- and dose-dependent increase in MKP5 mRNA that was dependent on VDR expression. Furthermore, VDR was found to associate with the VDRE in the MKP5 promoter on 1,25D treatment. These data support direct transcriptional activation of the *MKP5* gene by 1,25D. Consistent with increased levels of MKP5, 1,25D inhibited p38 phosphorylation. MKP5 siRNA blocked p38 inactivation by 1,25D, which further showed that MKP5 mediates p38 inactivation by 1,25D.

The ability of 1,25D to inactivate p38 led us to examine the regulation of IL-6, which is downstream of p38 activation (28), by 1,25D. Suppression of UV-stimulated IL-6 production by 1,25D was previously shown in keratinocytes (27) and we suspected that similar activity could be mediated by MKP5 in normal prostatic epithelial cells. Using MKP5 siRNA, we showed that 1,25D inhibited UV-stimulated p38 activity and IL-6 production in a MKP5-dependent manner in E-PZ cells. UV irradiation did not increase IL-6 promoter activity but did increase mRNA half-life, indicating posttranscriptional regulation of IL-6 mRNA expression. 1,25D pretreatment was able to attenuate the UV-stimulated increase in IL-6 mRNA half-life. Induction of IL-6 by a more physiologically relevant stress, TNF-α, was similarly inhibited by 1,25D.

IL-6 has been shown to be negatively regulated by androgens and the androgen receptor in murine bone marrow–derived fibroblasts (31). It is unlikely that androgen receptor contributes to IL-6 regulation observed in E-PZ cells because E-PZ cells are typical of prostatic basal epithelial cells and do not express androgen receptor (32). There is evidence of cross-talk and regulation of androgen receptor by vitamin D (1). In cells where androgen receptor and VDR are both expressed, such as the luminal cells of the prostate, it is possible that cooperation of the two pathways could facilitate a further decrease in IL-6 expression.

IL-6, as well as other interleukins and/or their receptors, are overexpressed in PCa tissue and/or serum of PCa patients (15, 33). Elevated serum levels of IL-6 and TNF-α are associated with aggressive pathology and decreased survival of PCa patients (16). Increased IL-6 staining is observed in malignant prostate tissue compared with adjacent normal tissue and IL-6 also contributes to the development of hormone-refractory cancer by androgen-independent activation of the androgen receptor (17). *In vitro*, the PCa cell lines DU 145, PC-3, and LNCaP all express the IL-6 receptor and are responsive to exogenous IL-6; however, DU 145 and PC-3 also greatly overexpress endogenous IL-6 whereas the LNCaP cell line does not express any IL-6 (15). The mechanism(s) leading to constitutive overexpression of IL-6 in PCa cells and

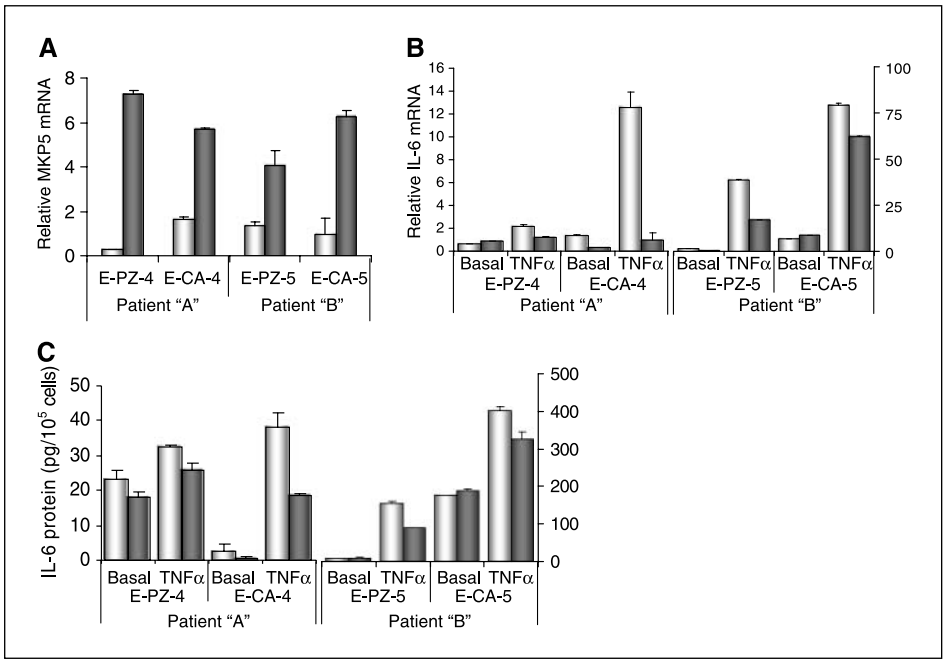

Figure 6. 1,25D up-regulates MKP5 mRNA and inhibits TNF-α-stimulated IL-6 production in matched pairs of normal and tumor cells from individual PCa patients. *A,* quantitative RT-PCR measurement of MKP5 mRNA levels in patient *A* E-PZ and E-CA cells and patient *B* E-PZ and E-CA cells 15 hours after treatment with vehicle (*open columns*) or 50 nmol/L 1,25D (*shaded columns*). *B,* quantitative RT-PCR analysis of IL-6 mRNA 3 hours after 50 ng/mL TNF-α in cells pretreated 15 hours with vehicle (*open columns*) or 50 nmol/L 1,25D (*shaded columns*). *MKP5* and *IL-6* gene expressions are shown relative to untreated control and normalized to expression of the housekeeping gene *TBP*. *Bars,* SD of triplicate samples. *C,* ELISA measurement of secreted IL-6 in cell culture media 3 hours after 50 ng/mL TNF-α in E-PZ and E-CA cells pretreated for 14 hours with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*).

tissues has not been fully characterized but seems to be transcriptional rather than posttranscriptional (34).

Although proinflammatory factors are overexpressed in PCa, it is unclear whether increased levels of these factors are required for development of the cancer originally or are a consequence of the cancer. On one hand, inflammation may trigger the initial expression of these factors in normal prostate tissue and when PCa arises, the PCa cells maintain these features for a survival advantage. On the other hand, because there is significant inflammatory infiltrate in PCa lesions, the possibility of the inflammatory genes becoming expressed later in PCa development cannot be excluded. If the former situation occurs, then our results suggest that the ability of 1,25D to suppress the synthesis of IL-6, and perhaps other inflammatory factors, may be a key component in blocking carcinogenic events associated with inflammation.

Another important finding from our study was that 1,25D up-regulated MKP5 in primary cultures derived from normal prostatic epithelium or primary adenocarcinomas of the prostate and in SV40 Tag–immortalized prostatic epithelial pRNS-1-1 cells, but not in the prostatic stromal cells or in PCa cell lines. pRNS-1-1 cells are not sensitive to growth inhibition by 1,25D yet retain functional VDR (1). Stromal cells (F-PZ) derived from normal prostatic tissue and PCa cell lines did not induce MKP5 when treated with 1,25D although these cells express VDR and are growth-inhibited by 1,25D (1). Furthermore, the squamous cell carcinoma cell lines A431 and SCC-25 displayed attenuated MKP5 up-regulation following 1,25D treatment compared with normal keratinocytes although they too retain VDR and growth inhibition by 1,25D (35, 36). We observed up-regulation of MKP5 by 1,25D in the matched pairs of E-PZ and E-CA cells from individual patients. These E-CA cells were derived from localized PCa whereas PC-3, DU 145, and LNCaP were all derived from PCa metastases. These data suggest that localized PCa may still be responsive to the anti-inflammatory properties of vitamin D. Together these observations show that (*a*) induction of MKP5 by

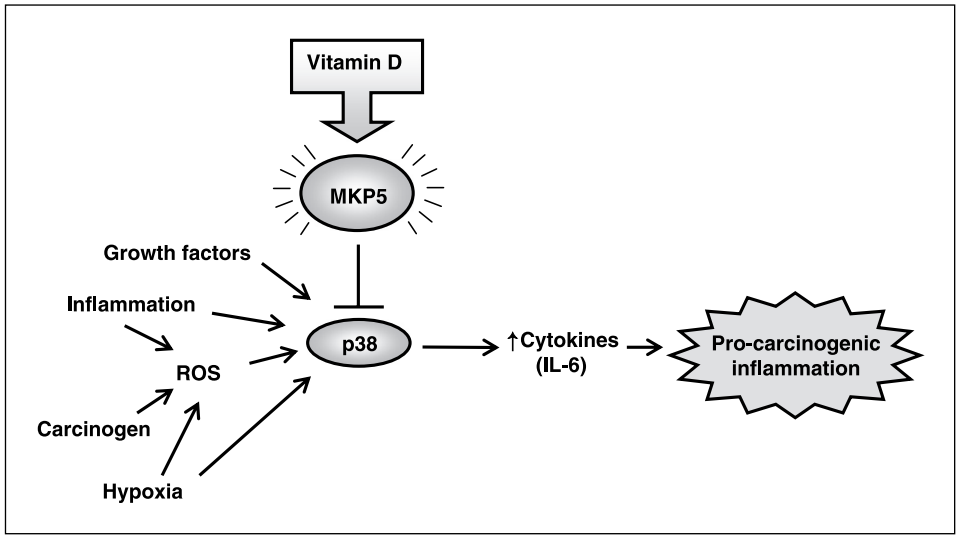

Figure 7. Proposed mechanism for anti-inflammatory activity and PCa prevention by vitamin D.

1,25D is specific to normal and localized malignant prostatic *epithelial* cells and does not occur in normal prostatic *stromal* cells; (*b*) induction of MKP5 is *independent* of growth inhibition by 1,25D; and (*c*) lack of 1,25D-induced MKP5 in PCa cell lines is not a result of immortalization per se because immortalized pRNS-1-1 cells up-regulate MKP5 in response to 1,25D.

The quantitative RT-PCR data show that the basal levels of MKP5 are lower in the PCa cell lines compared with normal prostatic epithelial cells. Because the established metastasis-derived PCa cell lines have low levels of MKP5 and are unable to induce MKP5 in response to 1,25D, it is tempting to speculate that loss of MKP5 may occur during PCa progression as the result of selective pressure to eliminate tumor suppressor activity of MKP5 and/or 1,25D. A number of MKP family members have been suggested to be tumor suppressors. In PCa MKP1 has been shown to be down-regulated (37). Candidate MKP tumor suppressors in other malignancies include MKP7, which is frequently deleted in lymphoblastic leukemia (38), MKP3, hypermethylated or deleted in pancreatic cancer (39, 40), and MKP2, which is deleted in breast carcinoma (41).

The link between inhibition of p38 by 1,25D via MKP5 and PCa prevention becomes more apparent when the many different ways the p38 pathway may become activated are considered. In addition to inflammatory cytokines, osmotic stress, UV irradiation (which we used in these studies), reactive oxygen species and hypoxia also activate p38 (42). Reactive oxygen species can amplify p38 activation because they are generated during hypoxia and as a by-product of inflammation (refs. 6, 8; Fig. 7). Hypoxia has been implicated in PCa metastasis and progression to androgen independence (6).

In addition to a potential role in PCa prevention, p38 inhibition by 1,25D via MKP5 may be farther reaching and mitigate activities of vitamin D in other tissues. Recent cDNA microarray analyses have shown MKP5 up-regulation by vitamin D in skin (43), colon (44), and ovarian cells (45). Furthermore, the overall immunomodulatory activity of vitamin D on the VDR-expressing cells of the innate and adaptive immune system (46) is highly similar to the immunomodulatory role of MKP5 that was shown by Zhang et al. (47) using MKP5 knockout mice. The mechanism by which vitamin D reduced IL-6 mRNA stability, through MKP5-mediated p38 inactivation, may also be responsible for down-regulation of other mRNAs. Activation of the p38 pathway causes a robust and rapid increase in inflammatory response proteins by mRNA stabilization and increased translation through AU-rich elements in the 3′ untranslated region (UTR; ref. 48). Posttranscriptional regulation of inflammatory genes is the basis for the anti-inflammatory activity of p38 inhibitors (48). Decreased mRNA stabilization by vitamin D has been shown to mediate down-regulation of granulocyte macrophage colony-stimulating factor (49) and this mechanism may decrease the stability of other AU-rich 3′-UTR–containing proinflammatory mRNAs by vitamin D.

It is becoming apparent that inflammation, both chronic and acute, contributes to PCa development. The epidemiologic evidence combined with the molecular pathogenesis of PCa supports this hypothesis (7). If inflammation is a significant risk factor for PCa, then PCa prevention will best be achieved with agents, such as vitamin D, which inhibit inflammation and/or decrease the cellular stress response that accompanies inflammation. Our study shows that MKP5 is a mediator of anti-inflammatory effects of 1,25D and suggests that vitamin D may play a significant role in PCa prevention by facilitating p38 inhibition and reduced IL-6 production in prostatic epithelial cells.

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Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells

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Keywords: prostate cancer, chemoprevention, p38, MKP5, COX-2, NFκB, vitamin D, curcumin, resveratrol, 6-gingerol

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ABSTRACT

As inflammation emerges as a risk factor for prostate cancer, there is potential for chemoprevention by anti-inflammatory agents. Dietary phytochemicals have been shown to have chemopreventive properties which may include anti-inflammatory activities. In this study, we demonstrate a role for mitogen activated protein kinase phosphatase-5 (MKP5) in mediating anti-inflammatory activities of the phytochemicals curcumin, resveratrol and [6]-gingerol. We utilized the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β to increase p38-dependent nuclear factor kappa-B (NFκB) activation and expression of pro-inflammatory genes cyclooxygenase (COX)-2, IL-6 and IL-8 in normal prostatic epithelial cells. MKP5 over-expression decreased cytokine-induced NFκB activation, COX-2, IL-6 and IL-8 in normal prostatic epithelial cells, suggesting potent anti-inflammatory activity of MKP5. Pre-treatment of cells with a p38 inhibitor mimicked the results observed with MKP5 over-expression, further implicating p38 inhibition as the main activity of MKP5. Curcumin, the phytochemical found in turmeric, up-regulated MKP5, subsequently decreasing cytokine-induced p38-dependent pro-inflammatory changes in normal prostatic epithelial cells. Resveratrol and [6]-gingerol, phytochemicals present in red wine and ginger, respectively, also up-regulated MKP5 in normal prostate epithelial cells. Moreover, we found that prostate cancer cell lines DU 145, PC-3, LNCaP and LAPC-4 retained the ability to up-regulate MKP5 following curcumin, resveratrol and [6]-gingerol exposure, suggesting utility of these phytochemicals in prostate cancer treatment. In summary, our findings show direct anti-inflammatory activity of MKP5 in prostate cells and suggest that up-regulation of MKP5 by phytochemicals may contribute to their chemopreventive actions by decreasing prostatic inflammation.

INTRODUCTION

As the carcinogenic processes that ultimately lead to prostate cancer (PCa) are being revealed, inflammation has emerged as a significant risk factor for PCa development [1]. Tissue inflammation can contribute to cancer development by inducing oxidative damage and promoting cell growth [2]. Inflammation has been shown to play a causal role in the initiation and/or progression of other cancers including liver, bladder and gastric cancers [2]. Evidence linking inflammation to PCa include i) regular administration of non-steroidal anti-inflammatory drugs (NSAIDS) significantly decreased PCa risk in older men by 60-80% [3,4], ii) men with chronic and/or acute inflammation of the prostate have an increased risk of developing PCa [1,5] and iii) inflammatory cytokines are over-expressed in serum and prostate tissue of PCa patients. Furthermore, De Marzo et al. have characterized and identified areas within the prostate of proliferative inflammatory atrophy (PIA), a possible precursor lesion to adenocarcinoma, suggesting a multi-step process of PCa development [1].

The long latency observed in PCa is unique among malignancies and should provide a lengthy window of opportunity for intervention by chemopreventive agents. The hope of PCa prevention by “natural” dietary products, also known as phytochemicals, has become increasingly popular and concordantly the mechanism(s) of action of phytochemicals has become an active area of research. With the accumulation of evidence linking inflammation to PCa, anti-inflammatory activities may be a significant mechanism by which phytochemicals decrease PCa risk.

We have previously characterized MAP kinase phosphatase 5 (MKP5) as a mediator of anti-inflammatory activities of vitamin D (1,25-dihydroxyvitamin D_3, 1,25D) in the prostate [6]. Although not typically considered a phytochemical, vitamin D is an essential part of the diet and has been implicated in the prevention of PCa by epidemiologic and laboratory studies [7]. cDNA microarray experiments originally identified MKP5 as a gene up-regulated by vitamin D in

prostate [8], skin [9], colon [10], and ovarian cells [11]. MKP5 is a member of the dual specificity MKP (DS-MKP) family of proteins that dephosphorylate mitogen activated protein kinases (MAPKs). MKP5 specifically dephosphorylates the stress activated protein kinases (SAPKs) p38 and JNK [12,13]. The MKP5 knockout mice revealed that MKP5 regulates both innate and adaptive immune responses *in vivo* [14].

The ability of MKP5 to inhibit p38 is of interest to PCa prevention since p38 signaling mediates pro-inflammatory responses in cells [15], potentially contributing to PCa development [1]. Downstream anti-inflammatory effects of p38 inhibition can include decreased activation of nuclear factor kappa B (NFκB), reduced cyclooxygenase-2 (COX-2) expression and decreased production of pro-inflammatory cytokines. Consistent with p38 inhibition, we found that up-regulation of MKP5 by 1,25D decreased p38 phosphorylation and subsequently reduced interleukin-6 (IL-6) production in prostatic epithelial cells derived from normal tissues and localized adenocarcinomas [6]. 1,25D also decreased COX-2 expression in prostate cells [16] and NFκB activity [17] in melanoma cell lines, effects potentially mediated by MKP5.

The anti-cancer activity of curcumin (diferloylmethane), a polyphenol from turmeric (*C.Longa* Linn), has been extensively studied and is well-established *in vitro* and *in vivo* [18]. In PCa cell lines, curcumin and analogs of curcumin inhibit growth, induce apoptosis and decrease androgen receptor signaling [18]. Anti-inflammatory properties of curcumin have been shown in keratinocytes in which curcumin inhibited p38 signaling and NFκB activation [19]. Furthermore, Yan et al. identified MKP5 as a gene up-regulated by curcumin in MDA-1986 squamous cell carcinoma cells [20]. Since curcumin has been shown to have anti-inflammatory activity and inflammation may be a risk factor for PCa, some of the chemopreventive activities attributed to curcumin may be a result of MKP5-mediated anti-inflammatory activity.

This aim of these experiments was to investigate the potentially chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MKP5 in prostate

cells. Using a pro-inflammatory cytokine stress, we demonstrated an anti-inflammatory role of MKP5 in normal prostatic epithelial cells by exogenous modulation of MKP5 levels with an expression vector and with siRNA. Curcumin up-regulated MKP5 and inhibited pro-inflammatory signaling in both normal prostatic epithelial cells and PCa cell lines. Lastly, we found that MKP5 was also up-regulated by the phytochemicals resveratrol and [6]-gingerol in normal prostatic epithelial cells and PCa cell lines.

MATERIALS AND METHODS

Cell Culture and Reagents

Human primary prostatic epithelial cells were derived from radical prostatectomy specimens. The patients did not have prior chemical, hormonal or radiation therapy. Histological characterization of the tissue of origin and culture of the prostate cells was as previously described [21]. Epithelial cells derived from the normal peripheral zone of the prostate (E-PZ) were cultured in supplemented MCDB 105 (Sigma-Aldrich, St. Louis, MO) or PFMR-4A as previously described [21]. Human PCa cell lines LNCaP, PC-3 and DU 145 were acquired from ATCC (Manassas, VA). LNCaP cells were cultured in MCDB 105/10 % fetal bovine serum (FBS), and PC-3 and DU 145 in DMEM (Invitrogen, Carlsbad, CA)/10 % FBS. LAPC-4 cells (provided by Dr. Charles Sawyers, UCLA, Los Angeles, CA) were cultured in phenol red-free RPMI 1640 (Life Technologies, Rockville, MD)/10 % FBS. All chemicals were obtained from Sigma-Aldrich unless otherwise noted. SB202190 was obtained from Calbiochem (San Diego, CA), reconstituted in dimethylsulfoxide (DMSO) and stored at -20ºC. Curcumin and gingerol (Wako Pure Chemicals, Osaka, Japan) were reconstituted in 5 % DMSO/95 % ethanol and stored at -20ºC. 1,25-dihydroxyvitamin D_3 (Biomol International, Plymouth Meeting, PA) and resveratrol (Calbiochem, La Jolla, CA) were reconstituted in 100 % ethanol and stored at -20ºC.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

RNA was isolated from cells by lysis in Trizol™ (Invitrogen) followed by chloroform extraction. The aqueous phase was then precipitated in 100% isopropanol and the pellet washed in 75% ethanol before resuspension in water. RNA concentration and quality were determined by absorbance ratio at 260/280 nm using a UV spectrophotometer. Total RNA (2 μg) was reverse-transcribed using Stratascript RT (Stratagene, La Jolla, CA). Resulting cDNA was used for

qPCR amplification with gene-specific primers and the Brilliant Sybr Green kit (Stratagene) in the MX3005-Pro thermocycler (Stratagene). PCR conditions for all primer sets were optimized and have similar amplification efficiency under the following thermocycler conditions: 95ºC 5′, 34X (95ºC 30″, 58ºC 30″, 72ºC 60″), 72ºC 5′. Relative mRNA levels were calculated from the point where each curve crossed the threshold line (Ct) using the equation: Rel. Value = $2^{-[Ct(control) - Ct(test)]test\ gene} / 2^{-[Ct(control) - Ct(test)]housekeeping\ gene}$ [22]. Reactions were performed in triplicate and the values normalized to the expression of the housekeeping gene TATA-box binding protein (TBP) [23]. Primer sets were TBP: 5'-tgctgagaagagtgtgctggag-3' and 5'-tctgaataggctgtggggtc-3'; MKP5: 5'-atcttgcccttcctgttcct-3' and 5'-attggtcgtttgcctttgac-3'; IL-6: 5'-gaagattccaaagatgtagccg-3' and 5'-tgttttctgccagtgcctc-3'; interleukin-8 (IL-8): 5'-agccttcctgatttctgcagctct-3' and 5'-aaacttctccacaaccctctgcac-3'; COX-2: 5'-gatactcaggcagagatgatctaccc-3' and 5'-agaccaggcaccagaccaaaga-3'; tumor necrosis factor-α (TNFα): 5'-aggcggtgcttgttcctcag-3' and 5'-tacaggcttgtcactcgggg-3'; interleukin-1β (IL-1β): 5'-ggcttattacagtggcaatgag-3' and 5'-tagtggtggtcggagattcgtagc-3'.

Cell lysate preparation and immunoblot

Cells were lysed in ice-cold 1X Cell Lysis Buffer (Cell Signaling, Beverly, MA) containing 1 μM phenylmethylsulfonyl fluoride (PMSF) and 100 nM okadaic acid. Cells were disrupted by sonication and insoluble cell debris removed by centrifugation at 15,000 x g, 4º C. Protein concentrations of the cell lysates were quantified using the BioRad Protein Dye (BioRad, Hercules, CA). Fresh cell lysates were used for analysis of phosphorylated proteins. Cell lysates (10-30 μg) were mixed with LDS NuPAGE Sample Buffer, separated by electrophoresis through 10% NuPAGE Bis-Tris Gels (Invitrogen) and transferred onto PVDF membrane. Membranes were probed with the following primary antibodies: anti-phospho-p38 rabbit polyclonal and anti-p38 rabbit polyclonal from Cell Signaling, monoclonal anti-COX2 (Cayman

Chemical, Ann Arbor, MI) and monoclonal anti-actin (Santa Cruz Biotech, Santa Cruz, CA). Following primary antibody incubation overnight at 4ºC, the blots were incubated with appropriate secondary horseradish peroxidase conjugated-antibodies (Cell Signaling) and developed with HyGlo ECL reagent (Denville Scientific, Metuchen, NJ).

siRNA transfection

Cells at 75% confluency in 60 mm collagen-coated dishes were transfected with 10 nmol of negative control (Ambion, Austin, TX) or MKP5-specific siRNA (Ambion) using 7 µl of siPORT NeoFX (Ambion) per dish, layering transfection mixture on top of the cells. Transfection reagent was not removed from the cells and cells were used for experiments after transfection as indicated in results and figure legends.

MKP5 over-expression

Full-length MKP5 cDNA (Genbank accession BC063826) was cloned into pcDNA3.1 (Invitrogen) using primers 5'-tgaatgtgcagagtccatagc-3' and 5'gttagcagggcaggtggtag-3' and confirmed by sequence analysis. pcDNA3.1/MKP5 or pcDNA3.1/LacZ were transiently transfected into 50% confluent E-PZ cells using siPORT NeoFX transfection reagent (Ambion). For Figure 2A, B, and D, 1 µg of plasmid DNA and 7 µl of siPORT NeoFX transfection reagent were used with 75 % confluent 60 mm collagen-coated dishes.

NFκB-Luciferase Reporter Activity Assay

NFκB-Luciferase construct (Clonetech, Mountain View, CA) containing four tandem copies of the NFκB consensus sequence driving luciferase expression and pRL-null-renilla (Promega, Madison, WI) were transiently transfected into E-PZ cells using siPORT NeoFX reagent. Cells (4.5×10^4 cells per well) were transfected in suspension with 10 ng of plasmid and 1 µl of NeoFX and plated onto 24-well collagen-coated plates. For Figure 2C, 250 and 500 ng of

pcDNA3.1 plasmid were included in the transfection. Eight hours after transfection, cells were treated as indicated in figure legends. Luciferase activity was measured using the Dual-luciferase Assay Kit (Promega). The ratio of luciferase to renilla-luciferase was determined to correct for transfection efficiency.

RESULTS

Characterization of TNFα and IL-1β as inducers of p38-dependent NFκB activation, COX-2 expression and pro-inflammatory cytokine production in normal prostatic E-PZ cells

To evaluate the role of MKP5 in the inflammatory response of normal prostatic epithelial cells derived from the peripheral zone (E-PZ), it was necessary to identify a pro-inflammatory stimulus that signaled through p38. TNF*α* and IL-1*β* are potent pro-inflammatory cytokines, secreted by macrophages to initiate inflammation by stimulating the expression of pro-inflammatory genes such as IL-6, IL-8 and COX-2 [24]. The pro-inflammatory cytokines IL-6 and IL-8 are of particular interest to PCa prevention because they are characteristically over-expressed in PCa tissue and patient serum [25]. COX-2 functions to increase prostaglandin secretion from prostate cells which can further facilitate inflammatory cell recruitment as well as increase cell proliferation [16]. IL-1β and TNFα also induce their own expression causing an amplification loop of the inflammatory response [24]. Importantly, TNF*α* and IL-1*β* have been shown to signal through both the p38 MAPK and the NFκB pathways [24,26].

E-PZ cells were stimulated with a combination of 1 ng/ml TNFα and 5 ng/ml IL-1β (T+I) for 15 minutes, and 1, 3, 6 and 24 hours. Cells were co-treated because TNFα and IL-1β showed a synergistic effect on p38 phosphorylation, COX-2 and inflammatory cytokine expression in the prostate cells (data not shown), which is similar to other published results [27,28]. Cell lysates and RNAs were collected at each time point. Immunoblot of cell lysates showed that phosphorylation of p38 occurred within 15 minutes of T+I treatment (**Figure 1A**) and increased COX-2 mRNA and protein occurred within 6 hours of T+I (**Figure 1A, B**). JNK phosphorylation was not stimulated by T+I in the E-PZ cells (data not shown). Since NFκB has been implicated in both TNF*α* and IL-1*β* signaling, NFκB activation by T+I was determined by luciferase enzyme activity of a transiently transfected NFκB-luc reporter construct. Twenty four hours following transfection with NFκB-luc and pRL-null, E-PZ cells were treated with T+I for 8

hours and luciferase activity was measured. T+I stimulation increased NFκB-luc activity 10-fold (**Figure 1C**). NFκB p65 nuclear translocation and activity are dependent upon phosphorylation and subsequent degradation of the inhibitory protein IκBα [29]. This is a point at which the p38 and NFκB pathways converge via p38-mediated phosphorylation of IκBα [30]. To test whether T+I –stimulated NFκB activity was dependent upon p38 signaling in the E-PZ cells, SB202190, a small molecule inhibitor of p38, was used. The results showed that in E-PZ cells T+I –stimulated NFκB activity was dependent upon p38 signaling as 1 hour pretreatment with 10 μM SB202190 completely blocked the increase in NFκB-luc activity (**Figure 1C**).

Increased mRNA accumulation of the pro-inflammatory cytokines IL-6, IL-8, IL-1β and TNFα can occur by two mechanisms: 1) increased p38-mediated mRNA stability and protein translation [31], and 2) increased NFκB-mediated gene transcription [32]. In E-PZ cells we observed increased mRNA accumulation of IL-6, IL-8, IL-1β and TNFα at 1 hour following T+I with maximum mRNA levels at 6 hours (**Figure 1D**). Levels of IL-1β and TNFα mRNA were measured as positive controls, because IL-1β and TNFα stimulate their own gene expression [24]. SB202190 was used to test whether cytokine mRNA stabilization was dependent upon p38 signaling. A 1 hour pretreatment with 10 μM SB202190 completely blocked the increase in cytokine mRNA by T+I (**Figure 1E**). MKP5 mRNA levels were unchanged by T+I (data not shown).

Therefore, T+I stimulation was used in all subsequent experiments because it caused a robust increase in cytokine mRNA, COX-2 mRNA and protein, and NFκB activity in E-PZ cells, all of which could be blocked by SB202190 and hence were p38-dependent.

MKP5 inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 expression and cytokine production in E-PZ cells

Our next goal was to assess whether MKP5, as a p38 MAP kinase phosphatase, could inhibit or decrease the powerful pro-inflammatory molecular changes induced by T+I stimulation in E-PZ cells. We previously showed that up-regulation of MKP5 by 1,25D attenuated UV-irradiation- and TNFα-induced p38 activation and IL-6 secretion in E-PZ cells [6].

To determine the role of MKP5 in the T+I response, the intracellular levels of MKP5 were up-regulated by an expression plasmid and down-regulated with siRNA. E-PZ cells were transiently transfected with pcDNA3.1-LacZ or pcDNA3.1-MKP5. Transfection with pcDNA3.1-MKP5 increased MKP5 mRNA levels 10-50 fold at 24 hours (data not shown). Twenty four hours following transfection, cells were stimulated with T+I for 15 minutes and p38 phosphorylation was measured by immunoblot of cell lysates. The cells transfected with pcDNA3.1-MKP5 had attenuated levels of phospho-p38 compared to pcDNA3.1LacZ, showing that the exogenous MKP5 was active (**Figure 2A**). Exogenous MKP5 was able to block T+I –stimulated COX-2 expression as pcDNA3.1-MKP5-transfected E-PZ cells showed less COX-2 protein by immunoblot analysis (**Figure 2B**). As a control, SB202190 was used and blocked basal and T+I –stimulated COX-2 protein expression (**Figure 2B**). Conversely, when MKP5 levels were decreased in E-PZ cells by siRNA, basal and T+I –induced COX-2 protein expression was increased compared to negative control siRNA (**Figure 2B**). MKP5-siRNA decreased MKP5 mRNA levels to 20 % of NEG-siRNA (data shown in **Figure 3C**). Since T+I -induced NFκB activation was dependent upon p38 signaling, the effect of exogenous MKP5 on NFκB-luc was measured. The luciferase assay showed that transfection of E-PZ cells with pcDNA3.1-MKP5 inhibited NFκB-luc activity in a dose dependent manner (**Figure 2C**). Consistent with decreased p38 signaling and NFκB activity, the levels of T+I –induced IL-6 and IL-8 were reduced more than 10-fold in E-PZ cells transfected with pcDNA3.1-MKP5 compared to control vector (**Figure 2D**).

These results reveal that MKP5 has a significant role in reducing T+I –stimulated inflammatory signaling by inhibiting p38-mediated pro-inflammatory processes in normal prostatic E-PZ cells.

MKP5 was up-regulated by curcumin and inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 up-regulation, NFκB activation and cytokine production in E-PZ cells

Curcumin is the polyphenolic compound present in the spice turmeric. Curcumin has been shown to have significant anti-inflammatory effects in a variety of cell types [19]. Interestingly, curcumin was found to up-regulate MKP5 mRNA in an MDA-1986 squamous cell carcinoma microarray study [20]. We speculated that curcumin would have similar anti-inflammatory effects in the normal prostatic E-PZ cells and that these effects would be mediated by MKP5.

In agreement with our hypothesis, curcumin dose-dependently up-regulated MKP5 mRNA (**Figure 3A**) and 17 hour pre-treatment with 50 μM curcumin inhibited T+I-stimulated p38 phosphorylation in E-PZ cells (**Figure 3B**). Curcumin did not affect mRNA stability of MKP5 as mRNA half-life following actinomycin D treatment was not different between vehicle and curcumin treated cells (unpublished results). Curcumin pre-treatment also blocked T+I –induced COX-2 protein expression and the effect was partially attenuated by MKP5-siRNA (**Figure 3C**). MKP5-siRNA attenuated MKP5 up-regulation by curcumin to 20 % of NEG-siRNA (**Figure 3C**). Downstream of p38 signaling by T+I, NFκB-luc activity and cytokine mRNA accumulation were blocked by 17 hour pre-treatment with 50 μM curcumin (**Figure 3D,E**).

These results show that MKP5 is up-regulated by curcumin and mediates anti-inflammatory activities of curcumin in normal prostatic E-PZ cells.

PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain ability to up-regulate MKP5 in response to curcumin

In our previous study we found that 1,25D up-regulated MKP5 only in primary E-PZ cells and cells cultured from primary adenocarcinomas of the prostate, but not in the PCa cell lines DU 145, PC-3 and LNCaP [6]. 1,25D typically alters gene expression through binding to the vitamin D receptor (VDR). VDR is a classical steroid receptor which translocates to the nucleus and binds to vitamin D response elements (VDREs) in the promoter regions of genes. This is the likely mechanism for regulation of MKP5 by 1,25D because the MKP5 promoter contains a putative VDRE and up-regulation of MKP5 by 1,25D was dependent upon VDR [6]. We did not find that curcumin up-regulated any of the classic VDR-regulated genes (data not shown) and this photochemical has not been reported to alter gene expression through VDR activation. The mechanism for MKP5 up-regulation by curcumin is probably distinct from that of 1,25D, and therefore MKP5 regulation by curcumin was examined in the immortalized PCa cell lines DU 145, PC-3, LNCaP and LAPC-4.

It is well established that curcumin can induce cell death in PCa cell lines at high concentrations [33]. After initially testing a range of curcumin concentrations (1-50 μM) (data not shown), we found that 25 μM curcumin was able to significantly up-regulate MKP5 mRNA in DU 145, PC-3, LNCaP and LAPC-4 cells (**Figure 4A**) without causing visible cell death (consistent with published data [33], therefore not shown). When stimulated with T+I, only the DU 145 cells responded with increased p38 phosphorylation and COX-2 expression. In the DU 145 cells, pre-treatment with 25 μM curcumin was able to block T+I –induced p38 phosphorylation and COX-2 protein expression (**Figure 4B**). Pre-treatment of DU 145 cells with curcumin decreased COX-2 mRNA similarly to SB202190 (**Figure 4B**), showing COX-2 expression was p38-dependent. T+I-induced NFκB-luc activity was not decreased by pre-treatment of DU 145 cells with either 10 μM SB202190 or 25 μM curcumin (**Figure 4C**). This finding correlates with published data showing that DU 145 cells have constitutively elevated

NFκB activity due to increased activity of IκB kinase (IKK) [34], which is p38-independent [30]. However, IL-6 and IL-8 cytokine mRNA levels were decreased by curcumin or SB202190 pre-treatment (**Figure 4D**), suggesting that T+I-induced expression of IL-6 and IL-8 is mediated by p38 rather than NFκB in DU 145 cells.

These results show that the PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain the ability to up-regulate MKP5 in response to curcumin and that curcumin reduced p38-mediated, but not NFκB-mediated, pro-inflammatory signaling by T+I in DU 145 cells.

MKP5 is up-regulated by the phytochemicals resveratrol and gingerol in normal prostatic E-PZ cells and PCa cell lines

Two other phytochemicals, resveratrol and 6-gingerol, have been shown to inhibit p38 phosphorylation and NFκB activation in mouse skin [35,36]. Since MKP5 was up-regulated by 1,25D and curcumin, by presumably different mechanisms, we wondered in MKP5 could be up-regulated and play a role in the anti-inflammatory activities of resveratrol and 6-gingerol in the prostate.

The ability of resveratrol and 6-gingerol to regulate MKP5 expression in normal prostatic E-PZ cells and in PCa cell lines was determined. In E-PZ cells we found that both 50 μM resveratrol and 50 μM 6-gingerol increased MKP5 mRNA to levels similar to those induced by 1,25D and curcumin (**Figure 5A**). With the exception of resveratrol in LNCaP cells, 50 μM resveratrol or 50 μM 6-gingerol was also able to up-regulate MKP5 in the PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 (**Figure 5B**).

These findings show that MKP5 is a common target of 1,25D and the phytochemicals curcumin, resveratrol and 6-gingerol in normal prostatic E-PZ cells, whereas in the PCa cell lines MKP5 is up-regulated by curcumin, resveratrol and 6-gingerol but not 1,25D.

DISCUSSION

We previously identified MKP5 as a mediator of inhibition of p38 and suppression of IL-6 by 1,25D in normal prostatic epithelial cells [6]. The current study expands on the inhibitory role of MKP5 in pro-inflammatory pathways in prostatic cells. Here, a combination of TNFα and IL-1β (T+I) was chosen as the pro-inflammatory stimulus in E-PZ cells because it rapidly increased COX-2 protein levels, NFκB activity and cytokine mRNA accumulation via a p38-dependent pathway. JNK activation was not involved in T+I -stimulated inflammatory signaling as JNK phophorylation was not detected. TNF*α* and IL-1*β* are also physiologically relevant to prostatic inflammation since they are secreted by resident macrophages and function as "alarm cytokines'" to initiate inflammatory cell recruitment by stimulating the expression of pro-inflammatory genes [24] (**Figure 6A**).

We focused on T+I-induced expression of the pro-inflammatory genes for COX-2 and the cytokines IL-6 and IL-8. COX-2 is the inducible form of cyclooxygenase, the rate-limiting enzyme in prostaglandin synthesis [16]. COX-2 expression results in prostaglandin secretion, which can increase cell proliferation and inflammatory cell recruitment [16]. The precise nature of COX-2 expression changes during PCa development and progression remain under debate. However, high COX-2 levels at radical prostatectomy are an independent predictor of PCa recurrence [37]. IL-6 and IL-8 are both found to be over-expressed in the tissues and serum of PCa patients [25,38,39]. Elevated serum levels of IL-6 are associated with aggressive pathology and decreased survival of PCa patients [40]. IL-8 has pro-angiogenic activity and is expressed by prostatic epithelial cells [41]. In vitro, the PCa –derived cell lines DU 145, PC-3 and LNCaP all express the IL-6 receptor and are responsive to exogenous IL-6. However, DU 145 and PC-3 also constitutively over-express IL-6 whereas the LNCaP cell line does not express any IL-6 [39]. Monoclonal antibody inhibition of IL-6 in LuCaP xenograft-bearing mice

blocked conversion to androgen-independence following castration [42]. Therefore, inhibition of IL-6 may prove to be a powerful tool in PCa treatment.

We showed that when MKP5 levels were increased in normal prostatic E-PZ cells by an MKP5 expression plasmid, pro-inflammatory effects of T+I stimulation were diminished. MKP5 decreased the levels of phosphorylated p38 protein, COX-2 protein, NFκB activation and cytokine (IL-6, IL-8, TNFα, IL-1β) mRNA accumulation. Pre-treatment of the cells with SB202190, a small molecule inhibitor of p38, mimicked the anti-inflammatory effects of MKP5 expression and verified p38 dependence of T+I-induced COX-2, NFκB activation and cytokine mRNA. Conversely, when levels of MKP5 were decreased by siRNA, the pro-inflammatory effects of T+I was exaggerated. These results reveal that MKP5 has a significant role in reducing T+I –stimulated inflammatory signaling in normal prostatic epithelial cells.

Curcumin, the phytochemical present in turmeric that is believed to have cancer preventive or therapeutic capabilities, had been shown to up-regulate MKP5 mRNA in MDA-1986 squamous carcinoma cells [20]. We showed that, as in MDA cells, curcumin was able to up-regulate MKP5 mRNA and inhibit p38 phosphorylation in normal prostatic E-PZ cells. Concordantly, the T+I -stimulated pro-inflammatory response in E-PZ cells was blunted by curcumin pretreatment. When siRNA was used to block the up-regulation of MKP5 by curcumin, COX-2 suppression by curcumin was attenuated, further implicating MKP5 as a mediator of curcumin's activity. T+I -stimulated NFκB activity was also blocked by curcumin. NFκB is a pro-inflammatory transcription factor whose regulation can be complex, involving many pathways. In the E-PZ cells, NFκB activation by T+I was completely p38 dependent and thus was inhibited by curcumin via MKP5. P38-dependent NFκB activation is not unique to the stimulus that we used, but also occurs during UV –irradiation and is IκB kinase (IKK)-independent [30]. These results show that MKP5 mediates anti-inflammatory activities of curcumin in normal prostatic E-PZ cells.

In our previous study, we observed that up-regulation of MKP5 by 1,25D was unique to prostate cells derived from normal tissues or localized adenocarcinomas [6]. In the metastases-derived PCa cell lines, MKP5 basal levels were lower and were unchanged by 1,25D treatment [6]. In our current study, we found that curcumin, in contrast to 1,25D, upregulated MKP5 in normal prostatic E-PZ cells as well as in the metastases-derived PCa cell lines DU 145, PC-3, LNCaP and LAPC-4. In order to explore the relevance of induction of MKP5 to anti-inflammatory activities of curcumin in the PCa cell lines, we evaluated the response of the cell lines to T+I. Stimulation of the PCa cell lines by T+I selectively stimulated p38 phosphorylation only in DU 145 cells. This was not unanticipated since both TNFα and IL-1β signal through receptor-mediated pathways that do not necessarily involve p38 MAPK [30]. In the DU 145 cells, curcumin decreased T+I-stimulated p38 phosphorylation, COX-2 protein expression, and IL-6 and IL-8 mRNA levels. Although DU 145 cells have constitutively activated NFκB [43], we found that T+I treatment further increased NFκB activity. However, activation of NFκB by T+I in the DU 145 cells, in contrast to E-PZ cells, was *p38-independent* as it was not blocked by curcumin or SB202190. Thus, although MKP5 is up-regulated by curcumin in the PCa cell lines, its activity is limited to p38-mediated effects on cytokine and COX-2 expression as NFκB activity is p38-independent in those cells.

Given the involvement of MKP5 in anti-inflammatory activities of two chemopreventive compounds, vitamin D and curcumin, we investigated several other phytochemicals suggested to have anti-cancer properties. Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a polyphenol present in grape skin and red wine [44]. Pezzuto et. al. have demonstrated chemopreventive activity of resveratrol in the initiation, promotion and progression stages of carcinogenesis [44]. In PCa cell lines, resveratrol inhibits cell growth and decreases androgen receptor signaling [45]. [6]-gingerol, the major pungent phenolic found in ginger (*Zingiber officinale* Roscoe, Zingiberaceae), has been utilized extensively in oriental medicine for alleviation of inflammation

and gastro-intestinal ailments [46]. However, prostate-specific anti-cancer activities of [6]-gingerol of have not yet been studied. Both resveratrol and [6]-gingerol inhibit phorbol ester-induced COX-2 expression and NFκB activation [35,36], activities we have shown can be mediated by MKP5. We found that resveratrol and [6]-gingerol, like vitamin D and curcumin, up-regulated MKP5 in normal prostatic E-PZ cells and in the PCa cell lines, with the exception of resveratrol in the LNCaP cells. In general, we found that LNCaP cells had limited ability to up-regulate MKP5 in response to any of the phytochemicals, suggesting the presence of genomic or epi-genomic suppression. MKP5 mRNA up-regulation by 1,25D and the phytochemicals is presumably via different mechanisms, as 1,25D altered MKP5 gene expression through the VDR [6] and the other phytochemicals do not. Although, our studies focused on mRNA regulation of MKP5, the possibility of further post-translational regulation of MKP5 activity cannot be excluded. Since MKP5 is a common target of these and potentially other phytochemicals, it is possible that combination dosing strategies could amplify MKP5 up-regulation, perhaps increasing anti-inflammatory activities while decreasing dosage of individual phytochemicals, thus decreasing side effects.

The fact that the PCa cell lines retain the ability to up-regulate MKP5 in response to phytochemicals is relevant to PCa therapy. It shows that not only can phytochemicals ingested in the diet and via supplements play a role in PCa prevention, but also that phytochemicals can be exploited for use in PCa treatment. P38 MAPK has diverse biological properties and activation can mediate apoptosis or cell survival, depending upon the type of stress, cell background and activities of the other MAPKs [15]. Therefore, whereas inhibition of the p38 pathway in normal tissue is an anti-inflammatory chemopreventive activity, p38 inhibition in PCa could affect cell survival pathways leading to increased sensitivity to chemotherapies. In fact, all of the phytochemicals we analyzed are currently under heavy investigation for their utility in cancer treatment and analogs with enhanced anti-tumor activity are being tested.

In conclusion, these experiments show that 1) MKP5 is a potent inhibitor of pro-inflammatory signaling in prostate cells and 2) MKP5 may be a common mediator of phytochemical anti-inflammatory activities. Our data suggest that, in the prostate, TNFα- and IL-1β-induced inflammatory cell recruitment could theoretically be decreased by phytochemical-induced MKP5 (**Figure 6B**), leading to a reduction in prostatic inflammation. Also, in contrast to 1,25D, which does not up-regulate MKP5 in PCa cell lines, curcumin, resveratrol and gingerol were also able to up-regulate MKP5 and inhibit the p38 pathway in the PCa cell lines, implicating potential utility in management of early or advanced PCa. Although we believe MKP5 to be an important mediator of inflammation in prostate cells, we are not down-playing other molecular effects of the phytochemicals. Utimately, phytochemicals have the ability to affect many pathways and a greater understanding of their mechanisms of action will facilitate exploitation of these naturally occurring “drugs” for cancer prevention and therapy.

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FIGURE LEGENDS

Fig. 1. Characterization of TNFα and IL-1β as inducers of p38-dependent NFκB activation, COX-2 expression and pro-inflammatory cytokine production in normal prostatic E-PZ cells. **A**, Immunoblot of E-PZ cell lysates following a time course of TNFα (1 ng/ml) and IL-1β (5 ng/ml) (T+I) treatment. Blot was probed with antibodies to phospho-p38, total p38, COX-2 and actin. **B**. Real-time qRT-PCR measurement of COX-2 mRNA following T+I treatment. **C**. Transactivation of NFκB-luc reporter construct 8 hours following T+I treatment of E-PZ cells pretreated for 30 minutes with vehicle (0.01 % DMSO) or 10 μM SB202190 (SB). Data is representative of three independent experiments; error bars represent standard deviation of triplicate samples. **D**. Real-time qRT-PCR measurement of IL-6, IL-8, IL-1β and TNFα mRNA in E-PZ cells after 1, 3, and 6 hours of T+I treatment. **E**. Real-time qRT-PCR measurement of IL-6, IL-8, IL-1β and TNFα mRNA following 3 hours of T+I treatment in E-PZ cells pretreated for 30 minutes with vehicle (0.01 % DMSO) or 10 μM SB202190 (SB). Dashed line represents basal mRNA levels for cytokines. For real-time qRT-PCR experiments mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TBP. Data is representative of three independent experiments; error bars represent standard deviation of duplicate or triplicate samples.

Fig. 2. MKP5 inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 expression and cytokine production in E-PZ cells. **A**. Immunoblot of E-PZ cell lysates following 30 minutes of TNFα and IL-1β (T+I) treatment. Cells were transfected with 1 μg pcDNA3.1 or pcDNA3.1-MKP5 24 hours prior to cytokine treatment. Blot was probed with antibodies to phospho-p38 and total p38. **B**. Immunoblot of E-PZ cell lysates following 24 hours of T+I treatment. Prior to cytokine treatment cells were either transfected with 1 μg pcDNA3.1 or pcDNA3.1-MKP5 for 24 hours, treated with 10 μM SB202190 for 30 minutes or transfected with 10 nmol of siRNA-NEG

or siRNA-MKP5. Blot was probed with antibodies to COX-2 and actin. **C**. Transactivation of NFκB-luc reporter construct in E-PZ cells 8 hours following T+I treatment. Cells were transfected 24 hours prior to cytokine treatment cells with NFκB-luc (10 ng), prL-null (10 ng) and pcDNA3.1 or pcDNA3.1-MKP5 (100 and 250 ng). Data is representative of two independent experiments; error bars represent standard deviation of triplicate samples. **D**. Real-time qRT-PCR measurement of IL-6 and IL-8 mRNA in E-PZ cells after 3 hours of T+I treatment. Cells were transfected with pcDNA3.1 or pcDNA3.1-MKP5 24 hours prior to cytokine treatment. For real-time qRT-PCR experiments mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TBP. Data is representative of three independent experiments; error bars represent standard deviation of duplicate or triplicate samples.

Fig. 3. MKP5 was up-regulated by curcumin and inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 up-regulation, NFκB activation and cytokine production in E-PZ cells. **A**. Real-time qRT-PCR measurement of MKP5 mRNA in E-PZ cells 17 hours after treatment with vehicle (0.01 % EtOH), 10 μM and 50 μM curcumin. **B**, Immunoblot of E-PZ cell lysates 30 minutes following TNFα and IL-1β (T+I) treatment. Blot was probed with antibodies to phospho-p38 and total p38. **C**. Immunoblot of E-PZ cell lysates 24 hours after T+I treatment in cells pre-treated with vehicle (0.01 % EtOH) or 50 μM curcumin. Four hours prior to pre-treatment, cells were transfected with 10 nmol of siRNA-NEG or siRNA-MKP5. Blot was probed with antibodies to COX-2 and actin. qRT-PCR measurement of MKP5 mRNA to show knockdown with siRNA-MKP5 relative to siRNA-NEG. **D**. Transactivation of NFκB-luc reporter construct in E-PZ cells, pretreated for 17 hours with vehicle (0.01 % EtOH), 10 μM and 50 μM curcumin, 8 hours following T+I treatment. Data is representative of two independent experiments; error bars represent standard deviation of triplicate samples. **E**. Real-time qRT-PCR measurement of IL-6 and IL-8 mRNA after 3 hours of T+I treatment in E-PZ cells, pretreated for 17 hours with vehicle

(0.01 % EtOH) or 50 μM curcumin. Dashed line represents basal mRNA levels for cytokines. For real-time qRT-PCR experiments mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TBP. Data is representative of three independent experiments; error bars represent standard deviation of duplicate or triplicate samples.

Fig. 4. PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain ability to up-regulate MKP5 in response to curcumin. **A**. Real-time qRT-PCR measurement of MKP5 mRNA in DU 145, PC-3, LNCaP and LAPC-4 following 17 hour treatment with vehicle (0.01 % EtOH) or 25 μM curcumin. **B**. Immunoblot of DU 145 cell lysates (10 μg) following 30 minutes and 24 hours of TNFα and IL-1β (T+I) treatment. Blots were probed with antibodies to phospho-p38, total p38, COX-2 and actin. Real-time qRT-PCR measurement COX-2 mRNA after 6 hours of T+I treatment in DU 145 cells, pretreated for 17 hours with vehicle (0.01 % DMSO) or 25 μM curcumin or 1 hour 10 μM SB202190. **C**. Transactivation of NFκB-luc reporter construct in DU 145 cells 8 hours following T+I treatment. Cells were pretreated for either 17 hours with vehicle (0.01 % EtOH) or 25 μM curcumin or 1 hour with 10 μM SB202190. Data is representative of two independent experiments; error bars represent standard deviation of triplicate samples. **D**. Real-time qRT-PCR measurement of IL-6 and IL-8 mRNA after 3 hours of T+I treatment in DU 145 cells, pretreated for 17 hours with vehicle (0.01 % EtOH) or 25 μM curcumin or 1 hour with 10 μM SB202190. For real-time qRT-PCR experiments mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TBP. Data is representative of three independent experiments; error bars represent standard deviation of duplicate or triplicate samples.

Fig. 5. MKP5 was up-regulated by the phytochemicals resveratrol and gingerol in normal prostatic E-PZ cells and PCa cell lines. **A**. Real-time qRT-PCR measurement of MKP5 mRNA

in E-PZ cells following 17 hour treatment with vehicle (0.01 % EtOH), 50 nM 1,25D, 50 μM curcumin, 25 μM resveratrol or 25 μM gingerol. **B**. Real-time qRT-PCR measurement of MKP5 mRNA in DU 145, PC-3, LNCaP and LAPC-4 following 17 hour treatment with vehicle (0.01 % EtOH) , 25 μM curcumin, 25 μM resveratrol or 25 μM gingerol. For real-time qRT-PCR experiments mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TBP. Data is representative of three independent experiments; error bars represent standard deviation of duplicate or triplicate samples.

Fig. 6. Diagram of hypothesized anti-inflammatory changes induced by phytochemicals and 1,25D in prostatic epithelial cells. **A**, Pro-inflammatory signaling in the normal prostate. TNFα and IL-1β bind their surface receptors, TNF receptor (TNFR), IL-1 receptors (IL-1Rs) and Toll-like receptors (TLRs), which signal through p38 MAPK and NFκB to increase expression of pro-inflammatory genes to facilitate inflammatory cell recruitment (adapted from www.cellsignal.com with incorporation of data from this study). Solid lines represent our findings and dashed lines represent known pathways that were not shown in our study. **B**. Model for disruption of pro-inflammatory signaling by phytochemicals and 1,25D via MKP5 in normal prostate.

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